THE LBM ANTISERUM POOLS METHOD FOR IDENTIFICATION OF ENTEROVIRUSES.

by K. A. LIM.

Thesis: Degree of Doctor of Medicine
University of Edinburgh
1972

To Joe and Mati

Carleton

C.E. and S.Y.

and Rose.

SUMMARY.

The thesis describes a method discovered in 1958 of pooling antisera to facilitate the identification of enteroviruses. The method has been recently adopted for preparation of diagnostic reagents to be distributed to competent investigators by the WHO.

The Enteroviruses comprise 64 serologic types and the identification of a virus isolate with an established type is by neutralization of the virus isolate by antiserum prepared against the prototype virus. The problem is where to begin with so many types to be dealt with.

Even in 1958 when the author encountered the problem, there were 24 typing antisera to be considered. The conventional method then, was to divide the typing antisera into groups and to test the virus isolate against pools of antisera in each group. If the virus isolate was neutralized by a pool it was identified by tests against each of the antisera comprising that pool.

To save time and labour the author devised a method of pooling the antisera in such a way that a virus isolate may be identified by simultaneous tests against a small number of pools. In this method the antisera were distributed to different combinations of antiserum pools so that the antiserum pool or pools that neutralized a virus isolate would be a combination of pools unique for a virus type. This enabled identification of the virus isolate with that type.

As a practical test of the method, monkey antisera prepared against 24 prototype enteroviruses were pooled in seven pools which were shown to give specific neutralization patterns when tested against the respective prototype viruses. The pools were then successfully used in the identification of a number of field virus isolates that had been obtained in Mexico and in Houston.

The method of combination antiserum pools can be applied successfully for the identification of enteroviruses (and of other viruses) provided that high-titred antisera are available which do not cause heterotypic neutralization or non-specific (cytotoxic) effects when pooled together. The time required for identifying a virus isolate can be halved if the confirmatory test with monovalent antiserum is omitted, and much labour and materials can be saved.

The method was reported jointly by Lim and Benyesh-Melnick (LBM) in 1960 and was modified as an "intersecting serum scheme" described by Schmidt, Guenther and Lennette in 1961. As enterovirus antisera became available in high titre and in quantity it was felt that they would be most helpfully used if made available to competent

investigators as ready prepared combination antiserum pools. A comparison of 8 antiserum pools made by the LBM method and 13 antiserum pools by the "intersecting serum scheme" method proved that the former was just as efficient in identifying field virus strains as the latter. The LBM method was therefore adopted for the preparation of sets of 8 LBM antiserum pools for the identification of enteroviruses. These have already been distributed to central laboratories in many parts of the world.

The problem presented by multiplicity of types is even more vexatious in the rhinoviruses, of which there are 91 serotypes, than in the enteroviruses. Though there were still technical problems to be solved in producing high-titred rhinovirus antisera, Gwaltney (1966) successfully used the "intersecting serum scheme" for identification (by a micro-neutralization test method) of virus isolates against 60 rhinovirus serotypes. Kenny (1970) compared the theoretical advantages of both the methods described and came to the conclusion that the LBM antiserum pools method could be advantageously applied for identification of rhinoviruses, providing the technical conditions were met.

The LBM antiserum pools method so facilitates the identification of picornaviruses (enteroviruses and rhinoviruses) that rapid progress in understanding of diseases caused by these viruses should be possible.

STATEMENT

re "The LBM Antiserum Pools Method for Identification of Enteroviruses", thesis submitted for the Degree of Doctor of Medicine, University of Edinburgh by K. A. Lim.

The thesis was composed unaided by the author. The experimental work on which it is based was performed by the author in 1958 when he was a Trainee in the Department of Epidemiology and Virology, Baylor University Medical College, Houston, Texas, U.S.A. The method was invented by the author and described and published jointly with Dr. Matilda Benyesh-Melnick who collaborated in the practical tests of the method. Much of this publication is incorporated in the thesis.

INTRODUCTION

The experimental work on which this thesis is based was done in 1968, when the author visited the Department of Virology, Baylor College of Medicine, Houston, U.S.A., to gain experience in poliomyelitis research. While attempting the identification of enteroviruses isolated in tissue culture, the author devised a method that reduced considerably the materials and labour required. This method was reported by Lim and Benyesh-Melnick (1960) in a publication entitled, "Typing of viruses by combinations of antiserum pools. Application to typing of enteroviruses (Coxsackie and ECHO)" (1). (see Appendix).

It has taken more than a decade for the method to become known and accepted by investigators in various parts of the world. Antisera to enteroviruses were initially available only for research purposes and many investigators had only limited quantities of antisera of low potency that they had made themselves. However, through the World Health Organisation, antisera to most of the known enteroviruses have been distributed widely, since; and recently, it has been agreed that antiserum pools for identification of enteroviruses prepared by the method of Lim and Benyesh-Melnick (LBM) should also be distributed to competent investigators (2). Its value having been proven, the author ventures to present an account of the LBM antiserum pools method in this thesis.

After giving a brief account of the enteroviruses and the problem of their identification the author will describe in detail the principles of the use of combinations of antiserum pools. The preparation of such pools and the tests carried out with them will then be described. In the concluding chapter the author will discuss the value of the work described, referring to the adoption and modification of his method by other investigators for the identification of enteroviruses as well as of rhinoviruses.

It is natural that this thesis should incorporate much of the publication mentioned (1) which is attached as an appendix, but the opportunity has been taken to bring the nomenclature into line with current usage. As the thesis may be read by those not specially acquainted with enteroviruses, some points have been elaborated for the sake of clarity. The author has also departed a little from the succinct style of the above publication, which was written for a rather select readership, to make the thesis more palatable.

ENTEROVIRUSES

The enterovirus group was established in 1957 (3), bringing together the polioviruses, the group A and B coxsackieviruses and the echoviruses. In 1963, the International Enterovirus Study Group created the picornavirus group, comprising ether-insensitive RNA viruses (4), of which enteroviruses formed one subgroup, and rhinoviruses the other, amongst picornaviruses of human origin. Enteroviruses are inhabitants of the human alimentary tract and as a group they are associated with a variety of clinical syndromes the most severe of which are those involving the central nervous system. For general accounts of the enteroviruses reference should be made to reviews and standard text-books on virology (4-9). It is sufficient for the purpose of this work to deal in detail only with the classification and identification of enteroviruses.

Table 1 shows the number of enteroviruses that have been recognised, classified in groups by their biological characteristics (10).

Table 1.

1) polioviruses, types 1 – 3
2) coxsackieviruses of group A, types 1 – 24
3) coxsackieviruses of group B, types 1 – 6
4) echoviruses, types 1 – 34.

Although this table indicates 67 enteroviruses types, the actual number of types recognised is 64; coxsackievirus A23 was deleted as it is identical with the previously described echovirus 9; echovirus 10 was deleted when this virus was transferred to the reovirus group; echovirus 28 was deleted when it was recognised as a rhinovirus; and echovirus 34 was deleted because it is related to coxsackievirus A24 as a prime strain, leaving 63 valid types from Table 1. The recently established enterovirus type 68 brings the total to 64 (11).

Most enteroviruses can be adapted to grow in tissue culture of primate epithelial cells in which cytopathogenic effects are caused. Notable exceptions are coxsackieviruses A1, A5, A6, A19 and A22 which have to be propagated in infant mice. Host range has been found to be an unreliable character for classification and it has been agreed that new enterovirus types that are recognised should be named enteroviruses, numbered serially from No. 68, and the biological groupings referred to in Table 1 should be ignored. Enterovirus 68 (Fermon virus) has been recognised since (11).

Enteroviruses are identified by specific serological responses in neutralization, haemagglutination-inhibition, complement-fixation and other antigen-antibody

reactions. By far the most useful test in practice is the neutralization test carried out in monolayers of tissue cultures in tubes. In such a test, the virus isolate to be identified is mixed with antiserum prepared against a prototype virus and inoculated into tissue cultures. Under appropriate controlled conditions such as concentration of virus and potency of antiserum used, the cytopathogenic effect caused normally by the virus is prevented by antiserum of the homologous type.

Only occasionally are antigenic cross-relationships between different enteroviruses detected by the neutralization test; coxsackievirus A3 crosses with A8, A 11 with A15, and A13 with A18; echovirus E1 with E8, E12 with E29 and E6 with E30. Difficulties encountered in identification because of such antigenic relationships can be overcome to some degree by using a higher concentration of antiserum than normally required, but, for convenience, it may be necessary to identify a virus isolate as one of two possible virus types, for example, echovirus 1-8, (i.e. echovirus 1 or echovirus 8).

The practical problem to be faced by the investigator in the identification of an enterovirus isolate is where to begin when testing it against antisera to the many possible types of enteroviruses that have been recognised. In 1958, when the experiments to be described were performed, the author had the task of testing a number of virus isolates against antisera that had been prepared against 24 established prototype enteroviruses. In such circumstances, the recommended procedure was to test a virus isolate against four or more pools of antisera each containing antisera to certain prototype viruses (12). If the virus isolate was neutralized by one of the antiserum pools, then it was tested against each of the components of that pool. This method of identification may be referred to as the two-stage method, and the time required was twice that required for one neutralization test experiment. The number of tissue cultures required was determined by the number of virus-antiserum mixtures to be tested which was the sum of the number of antiserum pools and the number of antisera in the pool that neutralized the virus isolate. In addition, controls of uninoculated cultures and cultures inoculated with dilutions of the virus were normally required in each experiment.

In order to save time and materials, the author devised a method of composing the antiserum pools in such a way that a virus isolate could be identified in one neutralization experiment. This one-stage method was possible if each typing antiserum was distributed to antiserum pools in a pattern that was unique for that antiserum. The method was described by the author as the combination antiserum pools method because the number of different patterns of neutralization that is given by N number of

antiserum pools is obtained by taking N items one at a time, two at a time, three at a time and so on. Thus, the antisera can be distributed, each to a pool, or each to two pools or each to three pools and so on. The number of possible patterns can be calculated as ${}_{N}C_{1}, {}_{N}C_{2}, {}_{N}C_{3}$, that is, N, $\frac{N(N-1)}{1x2}$, $\frac{N(N-1)(N-2)}{1x2x3}$, and so on.

The practical application of this method and its evaluation in actual use for the identification of viral isolates is described in the next section.

CONSTRUCTION OF COMBINATION POOLS

Tables 2-4 show distributional patterns for diagnostic schemes employing four, five or seven pools.

In the first scheme (Table 2) antisera to 14 prototype viruses are distributed in four pools so that the pattern of positive results obtainable in neutralization tests against any virus type shall be specific (see last column, Table 2). The 14 patterns are derived by taking four items one at a time, then two at a time and then three at a time.

In the scheme shown in Table 2, prototype antiserum 1 is distributed to pool A only, prototype antiserum 5 is distributed to pools A and B and prototype antiserum 11 is distributed to pools A, B and C. When a virus that is tested against the four pools is neutralized by pool A alone, it is identified as type 1; when it is neutralized by both pools A and B, it is identified as type 5; when it is neutralized by pools A, B and C, it is identified as type 11.

The diagnostic validity of a neutralizing pattern such as A, AB or ABC in the above examples, holds only in the absence of serological cross-reactions between the prototype viruses and for a true "isolated" test virus, i.e., a virus preparation not contaminated by virus of another type. Where an "isolate" contains two types of viruses the results obtained may be ambiguous and may lead to false interpretations. For example, in the four-pool scheme described, a mixture of type 1 and type 5 viruses would be neutralized by pool A and not by pool B, and the mixture would be indistinguishable from a virus preparation containing only type 1 virus. Similarly, a mixture of type 5 and type 8 viruses would be neutralized by pool A only and be identified mistakenly as a type 1 virus. Such ambiguous possibilities arise only if the virus specimen tested contains more than one virus and if in the distribution scheme more than one class of distributional pattern is used. In a 1-class distribution pattern, each serum is distributed to only one pool, in a 2-class pattern each serum is distributed to two pools, and so on. If only 2-class patterns are used, a virus preparation containing a mixture of two virus types, say, type 5 virus and type 6 virus, as in the above example, would be neutralized by one pool only, viz., pool B. The presence of a virus mixture would be clearly indicated and there is no ambiguity.

The 14 distribution patterns shown in Table 2 exhaust the capacity of a four-pool scheme. The terminal patterns, 0-class and 5-class are not used because it is desirable, in practice, that the pools shall give both positive and negative results against appropriate controls. If more than 14 types are to be covered the number of pools has to be

Table 2

Distribution pattern for combination pools;

(4-pool scheme for 14 types)

Pattern	Prototype antiserum:	Antiserum present in pools:				Neutralization pattern for identification of virus type
		A	B	C	D	
1-class	1	x				A
	2		x			B
	3			x		C
	4				x	D
2-class	5	x	x			AB
	6		x	x		BC
	7			x	x	CD
	8	x			x	AD
	9	x		x		AC
	10		x		x	BD
3-class	11	x	x	x		ABC
	12		x	x	x	BCD
	13	x		x	x	ACD
	14	x	x		x	ABD

Table 3

Five-pool scheme for 30 types

Pattern	Prototype antiserum :	Antiserum present in pools :					Neutralization pattern for identification of virus type
		A	B	C	D	E	
1-class	1	x					A
	2		x				B
	3			x			C
	4				x		D
	5					x	E
2-class	6	x	x				AB
	7		x	x			BC
	8			x	x		CD
	9				x	x	DE
	10	x				x	AE
	11	x		x			AC
	12		x		x		BD
	13			x		x	CE
	14	x			x		AD
	15		x			x	BE
3-class	16	x	x	x			ABC
	17		x	x	x		BCD
	18			x	x	x	CDE
	19	x			x	x	ADE
	20	x	x			x	ABE
	21	x	x		x		ABD
	22		x	x		x	BCE
	23	x		x	x		ACD
	24		x		x	x	BDE
	25	x		x		x	ACE
4-class	26	x	x	x	x		ABCD
	27		x	x	x	x	BCDE
	28	x		x	x	x	ACDE
	29	x	x		x	x	ABDE
	30	x	x	x		x	ABCE

increased.

The five-pool scheme shown in Table 3 is analogous to the four-pool scheme. By increasing the number of pools to five the number of patterns available is increased to 30 (ignoring the 0-class and 5-class patterns). However, the number of sera in each pool is increased from 7 to 15. Since neutralizing antibody of each serum in a pool is diluted by the other sera, in a pool of 15 undiluted sera the antibodies would be mutually diluted by the factor 1:15. If, as is sometimes the case, high serum concentrations cause non-specific effects, and the neutralization test technique requires that the serum concentration be not greater than 1:10, then the final dilutions of 15 sera in a pool must be at an average dilution greater than 1:150 with respect to antibody content. Where the potency of the antisera to be used does not permit of such dilution then the number of pools must be increased and the distribution patterns used restricted to the lower classes. Five sera distributed by a 1-class pattern contribute one serum to each pool, but five sera distributed by a 4-class pattern contribute four sera to each pool. The serum concentration is increased considerably in the latter instance as compared to the former.

The seven-pool scheme shown in Table 4 uses only 1-class, 2-class and some of the 3-class patterns possible. There are 10 sera in each pool and 35 virus types are catered for. (Part of the seven-pool scheme shown in Table 4 was employed for testing the combination serum pools method in the identification of enterovirus isolates, as described below.) It may be noted that unallocated patterns in a scheme can be used for the inclusion of additional antisera as new virus types are recognized. If a 3-class pattern is used, the number of sera in each pool would be increased by three sera for every seven additional types. Table 4 shows only 7 of the 35 3-class patterns available ($_7C_3 = 35$). In this example, the addition of 14 more types to the scheme would increase the number of sera in each pool from 10 to 16. The antibody dilution factor for undiluted serum in the pool would be 1:16, and if serum should be diluted 1:10 at least, the antibody dilution factor would be 1:160. Under such conditions the average titre of antiserum to be used must be greater than 1:3,200 assuming that 20 units of antiserum are employed in the tests. (One unit of antiserum is the highest dilution of serum that neutralizes 100 TCD_{50} of virus).

If it is desirable to restrict the number of sera in each pool, an existing seven-pool scheme can be extended to an eight-pool scheme by distributing seven sera into a new pool and one of these sera into each of the original seven pools. In this instance, a two-class pattern would be used and the number of sera in the original pools increased

Table 4

Seven-pool scheme for 35 types

Pattern	Prototype antiserum :	Antiserum present in pools :							Neutralization pattern for identification of virus type
		A	B	C	D	E	F	G	
1-class	1	x							A
	2		x						B
	3			x					C
	4				x				D
	5					x			E
	6						x		F
	7							x	G
2-class	8	x	x						AB
	9		x	x					BC
	10			x	x				CD
	11				x	x			DE
	12					x	x		EF
	13						x	x	FG
	14	x						x	AG
	15	x		x					AC
	16		x		x				BD
	17			x		x			CE
	18				x		x		DF
	19					x		x	EG
	20	x					x		AF
	21		x					x	BG
	22	x			x				AD
	23		x			x			BE
	24			x			x		CF
	25				x			x	DG
	26	x				x			AE
	27		x				x		BF
	28			x				x	CG
3-class	29	x		x		x			ACE
	30		x		x		x		BDF
	31			x		x		x	CEG
	32	x			x		x		ADF
	33		x			x		x	BEG
	34	x		x			x		ACF
	35		x		x			x	BDG

by one. This extension does not alter the distribution of the original sera and the interpretation of results obtained for the types of viruses originally catered for, a point which gives flexibility to the method. If the new antisera are added to pools already prepared, the resulting slight dilution of antisera in the pools can usually be ignored. Where the new antisera are to be included in pools to be prepared appropriate adjustments in dilution technique can be made easily.

The preparation of combination antiserum pools and their use is described in the next two sections.

TYPING OF ENTEROVIRUSES BY COMBINATION POOLS

Materials and Methods.

Sera. The sera listed in Tables 5 and 6 were, with exceptions mentioned in the next paragraph, reference monkey antisera issued by the Committee on Enteroviruses, National Foundation (3).

Coxsackievirus A9 serum was rabbit antiserum prepared by Microbiological Associates, Bethesda. Coxsackievirus B1 through B5 sera were monkey antisera prepared by Dr. Benyesh-Melnick in the Department of Virology, Baylor College of Medicine, Houston, by immunization with mouse torso strains. The antisera listed in Table 9 were rabbit antisera prepared by Microbiological Associates, Bethesda. Melnick's lactalbumin hydrolysate medium (M-H) was used as diluent for both sera and viruses. Before use, antiserum dilutions or antiserum pools were inactivated at 56° C for 30 mins.

Viruses. Prototype strains of the viruses used to test the antiserum pools are listed in the next to last column of Table 6. All had been passed in monkey kidney tissue culture. Fresh passages were used in these experiments.

Unidentified viruses newly isolated from two sources by Dr. Benyesh-Melnick were tested against the pools. Twenty-four had been isolated from children in Mexico, who had been fed live oral vaccine, and from their families; six had been isolated in Houston from cases of aseptic meningitis or poliomyelitis and their contacts. These virus isolates were not neutralizable by poliovirus antisera types 1, 2 and 3 or a pool of all three antisera in repeated experiments.

Neutralization tests. Neutralization tests were carried out by conventional methods in trypsinized primary monkey kidney tissue culture tubes (3, 13). 0.1 ml of virus suspension containing 100 TCD_{50} of virus was mixed with 0.1 ml of antiserum and the mixture incubated at 37° C for two hours. 0.2 ml of mixture was then inoculated into tissue culture tubes in triplicate and the tubes incubated at 37° C in roller drums. Cultures were examined daily for 10 days and cytopathogenic effects (CPE) observed were graded one to four plus. A test mixture was considered neutralized if not more than one of the three tubes inoculated showed two plus CPE or if not more than two tubes showed one plus CPE. The virus suspension was titrated at the same time and results of the tests were accepted if the dose of challenge virus used was in the range of 30 to 1000 TCD_{50}.

Combination serum pools. The antisera for 24 enterovirus types were distributed into seven pools, A to G, as shown in Tables 5 and 6. The distribution pattern was derived

Table 5

Composition of combination serum pool

Combination Pool	Antisera* Present in Pools								Number of Sera Per Pool
A	A9	E18	E2	E10	B1	E1	B3	E12	8
B	A9	E11	E5	E13	E19	B4	E3		7
C	E11	E14	E2	E6"	B2	B3	E8		7
D	E14	E15	E5	E7	B1	B5	E3	E12	8
E	E15	E16	E6"	E9	E19	E1	B3	E8	8
F	E16	E17	E7	E10	B4	E3	E12		7
G	E17	E18	E9	E13	B5	B2	E8		7

*A = Coxsackievirus A; B = Coxsackievirus B; E = Echovirus.

Table 6

Distribution of monkey typing sera in combination pools, and neutralization patterns obtained with prototype viruses.

Type Serum *	Final Dilution of Serum	Serum Pools +							Prototype Virus Tested	Neutralization pattern
		A	B	C	D	E	F	G		
A9	50	x	x						Grigg	AB
E11	50		x	x					Gregory	BC
E14	50			x	x				Tow	CD
E15	50				x	x			Charleston	DE
E16	50					x	x		Harrington	EF
E17	50						x	x	CHHE-29	FG
E18	50	x						x	Metcalf	AG
E2	100	x		x					Cornelis	AC
E5	100		x		x				Noyce	BD
E6"	32			x		x			D'Amori	CE
E7	100				x		x		Garnett	DF
E9	100					x		x	Bourne	EG
E10	100	x					x		Lang	AF
E13	100		x					x	Hamphill	BG
B1	32	x			x				Conn-5	AD
E19	50		x			x			Burke	BE
B5	200				x			x	Faulkner	DG
E1	200	x				x			Farouk	ABEG‡
B4	160		x				x		Texas-13	BF
B2	125			x				x	Ohio-1	CG
B3	320	x		x		x			Nancy	ACE
E3	500		x		x		x		Berardi	BDF
E8	500			x		x		x	Bryson	ACEG‡
E12	500	x			x		x		Travis	ADF
No. of sera in pool ...		8	7	7	8	8	7	7		
Serum concentration of pools		1:21	1:21	1:19	1:24	1:20	1:24	1:23		

* A = Coxsackievirus A; B = Coxsackievirus B; E = Echovirus.
+ x indicates inclusion of serum in pool named at top of column.
‡ See Text.
Charleston, 96-51 strain was the prototype virus used.

from Table 4. It was considered desirable to omit 1-class patterns from our scheme so as to reduce the chances of obtaining ambiguous results should more than one virus type be present in any test virus. Thus, the 24 patterns included 20 out of 21 possible 2-class patterns and 4 out of 35 possible 3-class patterns. (The omission of one 2-class pattern and its replacement by one 3-class pattern was arbitrary and without significance). Consequently, any virus of the types covered by the scheme should be neutralized by at least two pools and a neutralization result obtained against one pool only would indicate the virus tested was not "isolated". Poliovirus types were excluded from our scheme because so many isolates in the Baylor laboratory proved to be polioviruses that it was more economical to test new strains first against poliovirus antisera only than to include the latter in a general diagnostic scheme.

The final dilution of a serum in the pool (Table 6) was that recommended if the serum were to be used in a test by itself. This was usually 20 units, a unit of antiserum being the dilution that would neutralize 100 TCD_{50} of virus (3). With antisera of prototype viruses for which prime strains were known to exist, 100 antibody units were used because prime strains required for their neutralization relatively more antibody than the prototype strain (3).

In preparing the pools, a dilution of antiserum was first made which was 10 times stronger than the dilution recommended for typing. The appropriate "10 x concentrate" antisera were pooled as indicated in Tables 5 and 6 in 1.0 ml amounts and each pool adjusted to a final volume of 10 ml with diluent. The resultant pools contained the antisera in the desired dilutions. (In anticipation, it may be noted that Melnick's method (10) of preparing LBM combination pools is to add together calculated amounts of undiluted antisera and make up the final volume to one litre (!) with diluent. For example, if an antiserum had a neutralizing titre of 1:5,000, 20 units would be 1:250 and 4 ml of undiluted antiserum would be required for one litre of antiserum pool. In 1958, the author was working with pools of 10 and 50 ml volumes).

In Table 6, the antisera used are listed with those of low potency at the beginning and those of high potency at the end because it was desired that the higher titred antisera should be distributed evenly between the pools in 3-class patterns, thus reducing non-specific effects due to high serum concentration. However, the dilutions at which some antisera were eventually used had to be modified after preliminary experiments in which it was found that the working dilutions that had been recommended failed to give adequate neutralization of prototype strains.

The serum concentrations (as distinct from antibody concentration) of the pools varied between 1:19 and 1:24. At these concentrations the sera did not cause apparent non-specific effects. The reciprocal of the serum concentration of a pool was calculated by dividing the sum of the reciprocals of the dilutions of each of the antisera in the pool by the square of the number of antisera. Thus, the sum of the reciprocals of the dilutions of sera in pool A (see Table 6) was 50 + 50 + 100 + 100 + 32 + 200 + 320 + 500 = 1,352, which when divided by 64 (there being 8 sera in pool A) gave 21.1, the reciprocal of the serum concentration of pool A. The serum concentration was 1:21, approximately.

RESULTS

Prototype viruses.

When the prototype viruses listed in Table 6 were tested against the pools they were neutralized by pools which contained the corresponding antisera. For example, Grigg virus (coxsackievirus A9) had the neutralization pattern AB, i.e., it was neutralized by pools A and B which were the only pools containing homologous antiserum. Similarly, Nancy virus (coxsackievirus B3) had the neutralization pattern ACE. However, there were some anomalous results. Farouk virus (echovirus E1) was neutralized by pools A and E which contained the homologous antiserum, and also by pools B and C. The latter two results were attributable to the presence in pools B and G of echovirus E13 antiserum, the lot in use having antibodies to echovirus E1 as well (3). Thus, the use of a reagent with untoward behaviour did not vitiate the effectiveness of the method. For the diagnostic scheme that had been prepared, the neutralization pattern for echovirus E1 was ABEG although, in theory, it should have been merely AE. On the other hand, the echovirus E13 prototype strain tested against the pools was a new plaque purified strain kindly supplied by Dr. Hammon. Its neutralization pattern was BG.

Bryson virus (echovirus E8) was neutralized by pools C, E and G, which contained homologous antiserum and also by pool A. The latter result is attributable to the presence in pool A of echovirus E1 antiserum which is known to neutralize echovirus 8 (3). Thus, the neutralization pattern for echovirus E8 was ACEG. At the dilution used (1:500) echovirus E8 antiserum did not neutralize echovirus E1 as shown by failure of pool C to neutralize Farouk virus.

Although tests against echovirus E10, E17, E18 and E19 were not completely carried out, the satisfactory results obtained with the other 20 prototypes were considered adequate evidence for practical application of combination pools.

Because 1-class patterns were not used in the scheme, neutralization of a virus preparation by only one pool would have immediately indicated the presence of a mixture of viruses of the serotypes comprising that pool. Assuming that only two different viruses were present in a virus preparation that was neutralized by pool A, these two viruses could have been any pair of the 8 serotypes comprising pool A. There were, possible, 28 such pairs. One way of identifying the two viruses in a mixture was to plate the mixture in tissue culture under agar overlay and to pick out a few plaques for identification. Once one member of the pair had been identified the other could have been isolated by plating out the mixture again in agar overlay containing antiserum to

the identified virus. Any plaques that appeared should be of the other virus which could be propagated and identified in the usual way. The method of "plaquing" was simple but would have taken much time. Therefore, it was advantageous if a method could be devised for analysing mixtures of viruses neutralized by one pool without attempting re-isolation of each of the components by the plaque technique.

Experimental mixtures of viruses were made by mixing together equal parts of two virus preparations each containing 100 TCD_{50} per 0.1 ml. M-1 was a mixture of coxsackievirus B1 and echovirus E1. M-2 was a mixture of coxsackievirus B1 and echovirus E2. Both these mixtures were found to be neutralized by pool A only. The eight sera in pool A were then mixed in six different ways to form six subpools, A1, A2, A3 ... as shown in Table 7. The distribution pattern was obtained by dividing the eight sera into four pairs, arbitrarily, in the order listed in Table 5 and to combine the four pairs two at a time. Thus, the antiserum pairs were A9 and E18, E2 and E10, B1 and E1, and B3 and E12. Both M-1 and M-2 virus mixtures were then tested against the six subpools with results shown in Table 7. M-1 was neutralized by subpools A3, A4 and A5 and since antisera to coxsackievirus B1 and echovirus E1 were the only antisera to be distributed to precisely these three subpools, the viruses in M-1 could be identified as these two viruses. The pools B to G that did not neutralize M-1 had been saved. Coxsackievirus B1 was recovered from pool E which contained antisera to E1, and echovirus E1 was recovered from pool D which contained antiserum to B1.

M-1 virus mixture was of a pair of types that coincided with one of the four pairs into which the 8 serotypes had been arbitrarily divided. There were 24 other possible combinations, of which M-2 virus mixture was one, that did not coincide with one of the original four pairs. Consideration of the way the six subpools were prepared showed that any combination of two viruses out of the eight concerned must be neutralized by at least one antiserum subpool. This was because each of the four original antiserum pairs was associated in turn with the other antiserum pairs, and within the six subpools each of the 28 possible pairs must be found in at least one subpool. M-2 virus mixture was, in fact, neutralized by subpool A3 which indicated that it was a mixture of two of the four types in subpool A3. Since the pairs, E2-E10 and B1-E1, would have been neutralized by three pools instead of by one they could be ignored. By testing M-1 against mixtures of pairs of antisera comprising the other four possible pairs, M-2 was identified as a mixture of echovirus E2 and coxsackievirus B1 (see Table 7).

The method of analysis described was tedious and had one chance in 7 of identifying

Table 7

Neutralization of virus mixtures by subcombination pools.

Pool A Sera	Subpools (A)						Neutralization Patterns
	A1	A2	A3	A4	A5	A6	
A9, E18	x	x			x		A1, A2, A5
E2, E10		x	x			x	A2, A3, A6
B1, E1			x	x	x		A3, A4, A5
B3, E12	x			x		x	A1, A4, A6

Subpool A3 Sera	Subpools (A3)				Neutralization Pattern
	A3-1	A3-2	A3-3	A3-4	
E1, E10	x				A3-1
E1, E2		x			A3-2
B1, E2			x		A3-3
B1, E10				x	A3-4

x indicates inclusion of sera in pool named at top of column.

M-1 mixture gave neutralization pattern A3, A4, A5, identified as mixture of B1 and E1 viruses.

M-2 mixture gave neutralization patterns A3 and A3-3, identified as mixture of B1 and E2 viruses.

the viruses in one neutralization test against the six subpools, as with M-1 virus mixture. There was a 6/7 probability that further testing would be required, as with M-2. However, the procedure took less time than the plaque method which would have involved propagation of virus and determination of TCD_{50}. On the other hand the plaque method would have allowed the identification of viruses in a mixture even if more than two viruses were present; alternatively, the discovery that an "untypable" virus was present. "Plaque purification" is, indeed, demanded before a virus can be considered for naming as a new type.

Another method of resolving pairs of viruses that neutralized one pool only, would be to test the culture fluids from the tubes that showed CPE. In at least two of the six unneutralized tubes only one virus would be present, as was demonstrated above. However, there would be no way of telling which tubes these were unless observation of the tubes revealed differences in CPE that might have offered a clue.

Viral isolates.

To test the application of the method, 24 viruses isolated in Mexico which could not be neutralized by poliovirus antisera were tested against the pools A to G. Fourteen isolates were identified in a single experiment with these seven pools – eight as coxsackievirus B5, three as echovirus E1 and one each as echovirus E14, E15 and E18. The identifications were subsequently verified by tests against the respective homologous serum alone. Representative results are shown in Table 8.

Ten of the 24 isolates were not neutralized by any pool. For this there were four possible explanations:

a) The virus preparation tested contained an excessive proportion of inactive virus which bound antibody and diverted it from neutralizing active virus upon the titre of which the challenge dose of virus was based. Such an experimental error might be corrected if a fresh passage of the virus were used. Moreover, inhibition of neutralization by inactive virus could be aggravated if the antiserum used in the pools had fallen in titre from that upon which the working dilution (second column of Table 6) was calculated. It has also been shown recently that aggregation of virus used in test may affect neutralizing titres of antibody (2). This could result in apparent failure of neutralization.

b) The virus was of a type against which only antiserum of low titre was available (such as echovirus E4 antiserum) and thus was not suitable for inclusion in the scheme.

c) The virus was of a type not yet recognized and, therefore, its antiserum, even if available, was not included. It was also possible that the virus was not an enterovirus but a virus belonging to some other virus group, for example, adenoviruses, which could be isolated from the lower alimentary tract by the procedures adopted in isolation of the viruses being tested. In this regard, it should be noted that "echovirus E10" included in the pools was subsequently removed from the enterovirus group and assigned to a new group, the reovirus group.

d) The virus preparation contained more than one virus type and the antisera to these types were not together in any pool. An example of such a mixture of two viruses would be a mixture of coxsackievirus A9 and echovirus E14. Reference to the last column in Table 6 shows that antiserum to the first of

Table 8

Representative results of neutralization tests of Mexico and Houston isolates with combination pools

Source of Specimens	Virus Isolate No.	Neutralization Pattern	Identification
Mexico	1929	DG	Coxsackievirus B5
	2408	ABEG	Echovirus E1
	1923	CD	Echovirus E14
	3177	DE	Echovirus E15
	3295		Untypable
	3295*	ACE	Coxsackievirus B3
	2685	+	Untypable
	2685*	DG	Coxsackievirus B5
Houston	8-89	CG	Coxsackievirus B2
	8-139	BC	Echovirus E11
	8-77	EF	Echovirus E16

* Virus harvested from test against pool of polio 1, 2 and 3 sera.

+ Poliovirus type 2 recovered from fluids in these tests.

these two viruses was distributed to pools A and B while antiserum to the second virus was distributed to pools C and D. A mixture of these two viruses would not be neutralized by any pool.

The Mexican isolates were recovered in the course of studies on the use of oral poliovirus vaccine and it was reasonable to expect a high frequency of isolates containing both a poliovirus (of vaccine origin) and some other virus normally circulating in the community. Such a virus mixture would neither be neutralized by poliovirus antisera nor by the antiserum pools A to G. To meet this possibility, the ten isolates that could not typed in the first attempt were further investigated. The "isolates" were propagated again in tissue cultures in the presence of antisera to all three polioviruses, and then titrated and tested again against the pools A to G. This procedure served not only as a confirmatory test of the first attempt at identification, but also as a test with freshly passed virus. Representative results of the second attempt are also shown in Table 8 (strains 3295 and 2685). After passage through the poliovirus antisera, strain 2685 could be identified as coxsackievirus B5 which evidently was a type prevalent amongst the Mexico isolates. The culture tubes from the first test on strain 2685 all of which showed virus growth had been saved, and on testing the virus that grew in the presence of pools D and G which contained antiserum to coxsackievirus B5, it was found to be poliovirus P2. Strain 2685 was originally untypable because it was a mixture of poliovirus P2 and coxsackievirus B5.

Strain 3295, freshly passed in the presence of poliovirus antisera, was identified as coxsackievirus B3. The virus, which in the first identification attempt grew in the presence of coxsackievirus B3 antiserum in pools A, C and E, was re-examined and found to be neutralized by coxsackievirus B3 serum and not by poliovirus antisera. Strain 3295 was, therefore, a "typable" virus not identified in the first attempt because of experimental error, or perhaps, because coxsackieviruses, like echoviruses exist in nature as strains within a type. Strains of broader antigenicity than the prototype virus are more difficult to neutralize with prototype antisera and have been referred to as "prime" strains (3). In the same fashion, two more isolates were typed further, one as coxsackievirus B5 and the other as echovirus E18.

Six isolates (passed through poliovirus antisera) remained untypable by the pools for reasons already elaborated. These isolates were tested by Dr. Benyesh-Melnick, against the following antisera individually : echovirus types E4, 20, 22, 23, 24 and 25, and found not to be neutralized by such antisera. These isolates were, therefore, not

neutralizable by antisera then available or they contained unrecognized mixture of viruses. To resolve the latter possibility, passage of the "isolates" by the plaque technique could have been attempted, but this was outside the scope of the present work.

Eight viruses isolated in Houston gave the following results: four were identified as coxsackievirus B2, two as echovirus E11, two as echovirus E16 (see Table 8). All these identifications were verified by tests against homotypic antisera individually.

The experiments described were with monkey antisera except for coxsackievirus A9 serum which had been prepared in rabbits but was of sufficiently high titre to warrant its inclusion in the scheme. To confirm the practicability of combination pools, rabbit antisera that were available and were of adequate potency were used to make another diagnostic scheme using only four pools as shown in Table 9. In all instances, neutralization patterns obtained when prototype viruses were tested against the pools were in accord with the distribution of antisera in the pools. However, the serum concentrations of the pools was about 1:10 and this caused some non-specific effects in that all pools of sera delayed the growth of virus as compared with control virus grown in presence of normal serum in low concentration. Experiments with rabbit antisera were discontinued.

Table 9

Distribution of rabbit sera in combination pools and neutralization obtained with homologous prototype viruses

Type Serum *	Final Serum Dilution	Pools				Neutralization Pattern vs Homologous Virus
		A	B	C	D	
A 9	25	x				A
B 5	25		x			B
E 3	20			x		C
E14	20				x	D
E 1	30	x	x			AB
E 8	30		x	x		BC
E11	30			x	x	CD
E 5	50	x			x	AD
E 7	75	x		x		AC
E10	75		x		x	BD
E 2	100	x	x	x		ABC
E 6	100		x	x	x	BCD
E12	125	x		x	x	ACD
E 9	150	x	x		x	ABD
Serum concentration of pools		1:11	1:10	1:10	1:11	

* A = Coxsackievirus A; B = Coxsackievirus B; E = Echovirus.

DISCUSSION

Mater artium necessitas.

The method described, for using typing antisera in combination pools, reduced the number of tests that had to be done to identify an unknown virus and halved, approximately, the time required. Conservation of materials and labour in a busy laboratory was essential; thus, necessity was the mother of invention. Although it was applied for the identification of enteroviruses, the method is of general application, for example, for the identification of rhinoviruses, provided that the antisera do not give heterotypic neutralization and are available at potencies high enough to permit of mutual dilution. The higher the specificity and potency of antisera, the fewer the pools that need be used. The more the antigenic types to be considered the greater the economy that may be achieved.

It has been shown that it was possible to test a virus isolate against antisera to 24 of the established cytopathic enteroviruses, omitting only echovirus E4 for which high titred antiserum was not available. The 24 sera were distributed in seven combination pools, but reference to Table 4 will show that 35 antisera can be included in a seven-pool scheme, with 10 antisera per pool. If more than 35 types have to be considered, the scheme can be extended by using 3-class patterns not shown in Table 4 or by using an eight-pool scheme (2).

The possibility of more than one virus being present in an isolate was constantly kept in mind. Such a virus mixture can be very troublesome and the virus "isolate" is often reported as "untypable", the implication being that the virus "isolate" was unrelated to any of the established types. A satisfactory approach to the problem of a mixture of known viruses masquerading as a new virus would be to process all "untypable" viruses by the plaque technique before dismissing them as untypable, but this would be more complicated than working only with tissue culture tubes.

Consider only those instances where two viruses are present in such proportions that a dilution of the virus preparation giving 100 TCD_{50} of infective virus would contain demonstrable virus of both types. In a diagnostic scheme of 24 virus types, there are potentially 276 pairs of viruses. Approximately half of such pairs would be neutralized by one or two pools in the seven-pool scheme because their antisera happened to be in the same pool or pools, but the remaining pairs would not be neutralized by any pool. As the scheme does not use 1-class pattern, neutralization by one pool only would indicate the presence of mixed viruses. Neutralization by two pools, however, may in

some combinations give rise to false identification. For example, a mixture of echovirus E3 and echovirus E12 would be neutralized by pools D and F, both these pools being the ones to contain antisera to both viruses. Such a neutralization pattern would be interpreted as neutralization of echovirus E7, (see Table 6, last column), and in fact any mixture of two of the three viruses, echovirus E3, E7 and E12, would give the neutralization pattern DF indicating neutralization of echovirus E7. Awareness of such a possibility prompts the precaution to be taken, that where a virus isolate has been identified as echovirus E7, it should be confirmed by testing against homotypic antiserum.

These ambiguous possibilities arise only because both 2-class and 3-class patterns are used; as only a few of the latter are used the number of ambiguous patterns possible are small and can be discern by inspection of table 6. Of the types identified in practice (see Table 8), only coxsackievirus B2 had a neutralization pattern (CG) which could be simulated by a mixture of coxsackievirus B2 and echovirus E8 (neutralization pattern : ACEG). All three identifications of coxsackievirus B2 were, in fact, verified by homotypic tests.

The important role the method of Lim and Benyesh-Melnick (LBM) could play in the study of enteroviruses was envisaged in the following lines they wrote in 1959 (1). "As the number of types in the enterovirus classification scheme increases, it becomes more and more impracticable for all laboratories to contemplate identification by conventional methods of the agents they isolate in tissue culture. There is a need, however, for the role which these viruses play in the etiology of disease to be studied as widely as possible. The gap between what is practicable and what is desired might be bridged by application of the method described. Provided that specific antisera of adequate titres were available, it is possible for a central laboratory to supply other laboratories with pools of sera made and tested in bulk. Without the necessity of having to carry stocks of antisera to all types, any laboratory can type virus isolates by tests against seven pools of sera, interpreting their results by means of a diagnostic table".

If a man can make a better mouse-trap ...*

An example of the "conventional methods" referred to in the previous paragraph, was the two-stage method (12) used by many laboratories, including the Baylor laboratory in 1958, and still recommended by manuals for clinical virology published in 1967 (14, 15). The combination pools method was devised to facilitate the identification of virus isolates against 24 serotypes, a number of types which entailed considerable labour and the use of much materials when typing was done by "conventional methods". By 1971 there were recognized 64 enteroviruses, and antisera to 60 serotypes, most of which were cytopathogenic, were available in quantity. The task of identifying virus isolates by the two-stage method against the larger number of serotypes would be even more laborious. The discovery of the one-stage, combination pools method was, therefore, timely and it was soon adopted for routine use in the WHO World Enterovirus Centre, at Baylor Medical College, Houston (10), as well as by some other investigators (16, 17, 18). It was also referred to in a paper by Koroleva and Voroshilova, "Human Enteroviruses" in "Virus and Viral Diseases", V.2, Moscow 1964, and in "Methods of Laboratory Diagnosis of Enterovirus Infections", by Voroshilova, Zhevandrova, and Balayan, Moscow, Medizina, 1964 (Prof. M. K. Voroshilova, Institute of Poliomyelitis and Viral Encephalitis, Moscow, personal communication).

Meanwhile, the report of Lim and Benyesh-Melnick (1) of their method which is now referred to as the "LBM antiserum pools method", was followed by its modification as an "intersecting serum scheme" method, reported by Schmidt, Guenther and Lennette in 1961 (19). This method was simpler than the LBM pools method and was adopted by those authors as well as by a number of other investigators (20, 21, 22). Lennette and his associates enjoyed a high reputation amongst investigators as compared with the

* "If a man can write a better book, preach a better sermon, or make a better mouse-trap than his neighbour, though he builds his house in the woods, the world will make a beaten path to his door". (Ralph Waldo Emerson, 1871).

relatively unknown Lim and Benyesh-Melnick, and the "intersecting serum scheme" introduced many investigators to the joys of a one-stage method of typing enteroviruses and rhinoviruses.

In the 3rd Edition, (1964) of that well-known laboratory manual, "Diagnostic Procedures for Viral and Rickettsial Diseases", Melnick, Wenner, and Rosen, in their chapter on the Enteroviruses (23) referred briefly to both the LBM pools method and the "intersecting serum scheme" without giving details. In the same book, Schmidt, in her chapter on Tissue Culture Methods (24), gave details of the "intersecting serum scheme", and Wenner in his chapter on Diagnosis of Enterovirus Infections (25), recommended its use. Antiserum pools made according to the "intersecting serum scheme" were distributed by WHO to a number of laboratories in the late 1960s, removing the need for any competent investigator to prepare such antiserum pools himself. However, by its very simplicity, the "intersecting serum scheme" had its drawback in that it was not an economical way of using the combination pools method.

Inspiration and perspiration*

In the "intersecting serum scheme" the antisera were distributed to pools in such a way that the serotypes could be arranged in rows and columns in a rectangle as shown in Table 10, adapted from Schmidt (24). For example, echovirus 12 serum was placed in row 3, i.e., pool 3, and also in column 2, i.e., pool 7. A virus neutralized by two pools (for example, pools 3 and 7) was identifed as the serotype standing at the intersections of the two pools (in this example, echovirus E12). The "intersecting serum scheme" used only 2-class patterns, and only part of the 45 possible 2-class patterns obtainable with 10 pools. Table 11 shows how the scheme shown in Table 10 can be extended to cover the maximum number of serotypes with 10 pools, with some sacrifice of elegance. It is still an "intersecting serum scheme" but turns out to be another way of presenting the 2-class patterns in the LBM pools method.

* "Genius is one per cent inspiration and ninety-nine per cent perspiration!" (Thomas A. Edison, quoted in Golden Book, April, 1931).

Table 10

Ten-pool intersecting serum scheme for identification of echovirus. (from Schmidt (18)).

Serum pool Nos.	6	7	8	9	10
1	E 1	E 2	E 3	E 4	E 5
2	E 6	E 7	E 8	E 9	E10
3	E11	E12	E13	E14	E15
4	E16	E17	E18	E19	E20
5	E21	E22	E23	E24	E25

E = Echovirus antiserum

Example, E12 virus is neutralized by pool No. 3 (3rd row) and pool No. 7 (2nd column).

Table 11

Extension of Ten-pool "Intersecting Serum Scheme"
for 25 enteroviruses to cover 45 enteroviruses,
(i.e. all possible combinations, two at a time,
of 10 pools)

2	3	4	5	6	7	8	9	10	Serum pool Nos.
E26	E27	E28	E29	E1	E2	E3	E4	E5	1
	E30	E31	E32	E6	E7	E8	E9	E10	2
		E33	E34	E11	E12	E13	E14	E15	3
			E35	E16	E17	E18	E19	E20	4
				E21	E22	E23	E24	E25	5
					E36	E37	E38	E39	6
						E40	E41	E42	7
							E43	E44	8
								E45	9

Examples:

E30 virus is neutralized by pools 2 and 3;

E44 virus by pools 8 and 10.

The simplicity of the "intersecting serum scheme" (Table 10) was attractive as long as investigators had to make up their own serum pools. If combination pools are supplied from a central source with a diagnostic key, how the pools are prepared need not concern the investigator. Considerable economy can then be achieved by using LBM pools with 1-class and 3-class patterns as well as all available 2-class patterns. To cover 42 serotypes in an "intersecting serum scheme" requires 13 antiserum pools, (the 5 x 5 scheme shown in Table 10 being replaced by a 6 x 7 scheme), and involves the use of 62.5% more materials as well as more effort in neutralization tests as compared with using 8 LBM antiserum pools. The conclusion that the LBM antiserum pools method was more efficient was confirmed in a study undertaken by three laboratories to evaluate the use of antiserum pools in the identification of field isolates (Schmidt et al. 1971, (2)). (See Appendix II)

The antiserum pools tested in the above trial were prepared both by the "intersecting serum scheme" method and by the Lim and Benyesh-Melnick method and it is significant that the diagnostic key given for the former pools was presented in tabular form rather than in the "intersecting" form. Schmidt et al., 1971, (2) made some important observations on problems encountered in the use of LBM antiserum pools and recommended that 50 antiserum units be used rather than 20 units hitherto recommended, and that the challenge dose of the test virus should be in the range of 320 to 3200 TCD_{50}. These modifications, on the one hand, prevented "break-through" of virus, i.e., delayed appearance of CPE caused by partially neutralized virus which could be interpreted as failure of neutralization, and, on the other hand, reduced the occurrence of heterotypic neutralization which would result in false identifications. It was found that practically as many enterovirus field strains could be correctly identified when 8 LBM antiserum pools were used as when 13 "intersecting serum scheme" pools were used, (92% as compared with 94% of 312 field strains), and the advantage of performing 8 tests rather than 13 tests was clearly demonstrated. As a result, through the World Health Organization, sets of 8 LBM pools have been prepared and distributed through the World Health Organization to national laboratories in many countries for use by competent investigators (Dr. J. L. Melnick, personal communication).

The LBM antiserum pools method has, indeed, bridged the gap between desire and fulfilment in the identification of enteroviruses, for any investigator may confidently approach the problem of identifying enterovirus isolates with 8 LBM antiserum pools without having to carry antisera to all enterovirus types. Substantial saving is achieved

because the investigator need not prepare working dilutions of each typing serum as he should sans LBM.

The greatest saving is in the time required to identify a virus. As compared to the older two-stage method, the LBM antiserum pools enabled the identification of enterovirus isolates in one run, halving the time required, if confirmatory tests with monovalent antiserum is deferred or omitted. In many epidemiologic studies, occasional errors in identification of a few virus isolates amongst hundreds are not of great consequence and an investigator who does not have monovalent antisera for confirmatory tests may refer strains of special interest to a central laboratory. This procedure has the pragmatic importance of enlisting the interest of many more investigators in problems in which they can play significant roles, while the central laboratory deploys its energies and facilities to the best advantage as adjudicator of problems that cannot be solved by peripheral laboratories.

The enterovirus antisera were produced in horses by the Research Reference Reagents Branch of the U.S. Institutes of Health, and their sensitivity and specificity were assayed in collaborative studies performed by WHO Regional Reference Centres and WHO Virus Collaborating Laboratories (26, 27, 28, 29, 30). The author was privileged to participate in these studies in association with his colleague, Dr. L. H. Lee, of the Department of Bacteriology, University of Singapore, also designated the WHO Regional Enterovirus Centre in Singapore. Although LBM pools at present cover 42 serotypes (2), there are already tested, antisera to 60 types (30), and the scope of the 8 LBM pools can be extended to cover all enterovirus types with at most two additional pools. More than 20 litres of horse antisera for each enterovirus type have now been prepared and there should be sufficient material to last, with judicious use, until the 21st century. To those who made the enterovirus antisera in quantity and to the international team of collaborators who tested the antisera and put them to practical use must go the credit for validating the idea of the LBM pools. If the author's contribution was a little inspiration, theirs was much perspiration.

Caviare*

This thesis is mainly concerned with the application of a one-stage combination pools method for typing of enteroviruses. Attention should be drawn, however, to the possibility of applying the method in other classification schemes where the conditions for success can be met. One instance where a labour saving device would be obviously helpful is the Rhinovirus group in which over 90 prototypes were listed in 1971 (11).

Whereas the enteroviruses were divided into subgroups and some investigators concerned themselves only with echoviruses, thus apparently limiting the number of types to be considered, the designation of rhinovirus types by numbers only, e.g., rhinovirus 1, 2, 3 ..., had been adopted several years before a similar classification procedure was adopted for enteroviruses. The problem presented by multiplicity of types was, therefore, more acutely felt by investigators concerned with rhinoviruses and they were not slow in exploring the method of combination pools for the identification of rhinoviruses.

The technical problems encountered with rhinoviruses were greater than with enteroviruses in that antisera to rhinoviruses previously available were of low potency and the tissue culture techniques used for rhinoviruses were somewhat more difficult than those for enteroviruses, but the prospect that combination pools might enable considerable saving of time and labour was so attractive that efforts were made to adapt the method for identification of rhinoviruses. Hamparian at el. in 1964 (31) used the "intersecting serum scheme" for screening rhinovirus isolates against 20 antisera to rhinovirus types. Gwaltney in 1966 (32) described a new method of micro-neutralization test for rhinoviruses, and compared its efficacy with that of the conventional macro-neutralization test in identification of rhinovirus isolates, using antiserum pools combined by the "intersecting serum scheme". Gwaltney was dealing with 60 rhinoviruses antisera and combined them in three "intersecting serum schemes"

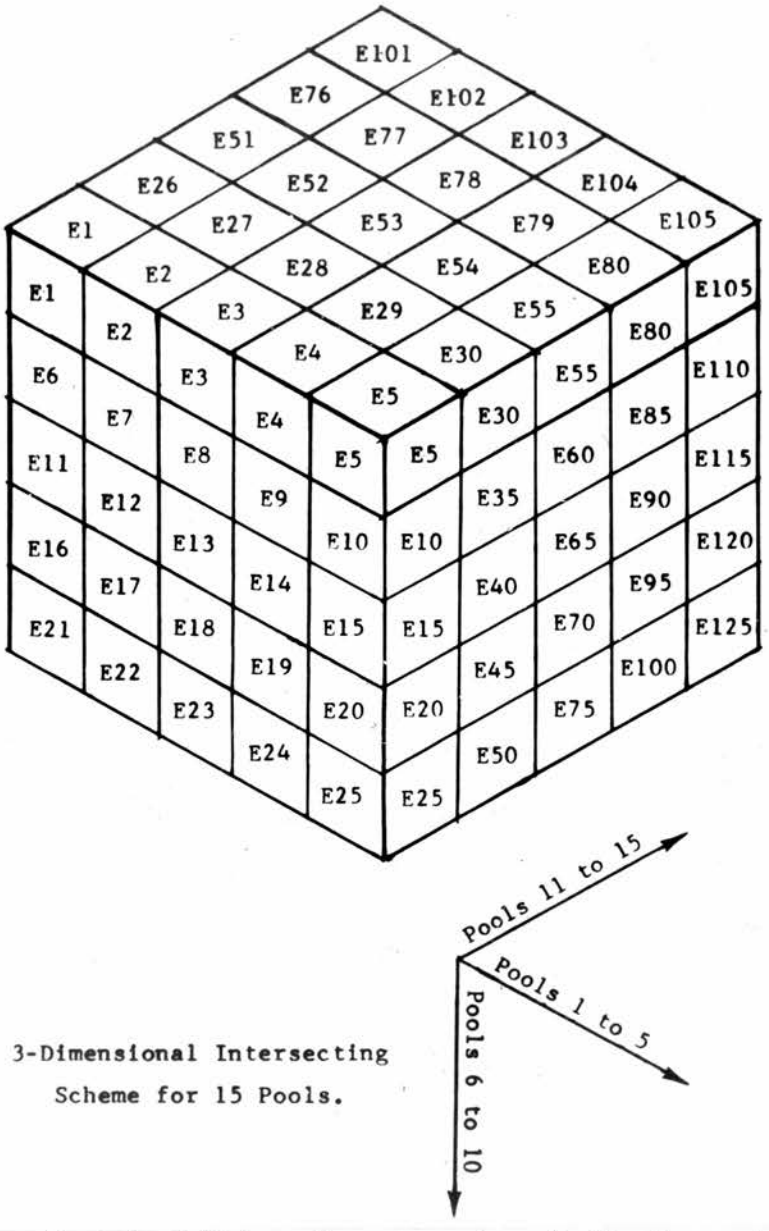
* "The play, I remember, pleased not the million; t'was caviare to the general". (Shakespeare, Hamlet. Act ii, sc. 2, l. 457).

of 20 antisera each, and although he did not mention the number of pools he used, presumeably there were nine pools in each scheme, making a total of 27 pools. Gwaltney was apparently content with the economies achieved by using a one-stage identification method, and he was, perhaps, more interested in demonstrating the superiority of his micro-neutralization test which cost one-ninth that of the conventional macro-neutralization test and, on average, took half the time to complete. As he did not cite the report of Lim and Benyesh-Melnick (1) he was probably unaware that a further systemic economy, as distinct from a comparative economy, might have been achieved by adopting the LBM pools method. This was a pity, for Gwaltney had overcome the problem of cytotoxicity due to high concentration of serum (calf serum of tissue culture media as well as antisera) by refeeding tissue cultures two hours after inoculation with virus-antiserum mixtures.

The interest shown by rhinovirus investigators in combination pools was further indicated by the report by Kenny et al. in 1970 (33) of "An analysis of serum pooling schemes for identification of large numbers of viruses". These authors discussed the factors involved in the neutralization test with serum pools, viz., T, the number of virus types which can be identified; P, the number of pools required by the identification scheme; S, the number of antisera included in each scheme; d, the dimension or side of square, cube or hypercube; and n, the number of dimensions. They gave an example in which n = 3 (three dimensional system) and stated that 125 virus types could be identified by 15 pools each containing 25 antisera per pool.

It is apparent that these authors sought to extend the "intersecting serum scheme" of Schmidt et al. (19) which was two dimensional, by distributing the antisera in a cubic pattern. Thus, if we imagine a cube of 5 x 5 x 5 compartments and number them along three orthogonal axes; pools 1 to 5 along one axis, pools 6 to 10 along another, and pools 11 to 15 along the third axis, then the location of a compartment, or type, is determined by the three reference co-ordinates, one along each axis. The number of antisera in each pool would be 25, a pool being represented by a slice of the cube representing 1 x 5 x 5 compartments (See Figure 1).

Kenny et al. (33) apparently overlooked the point that the "intersecting serum scheme" of Schmidt et al. (19) was a special case of the combination pools system for they distinguished between "dimensional schemes" and "combinatorial systems". In fact, 2-class patterns applied to 15 pools will allow the identification of 105 types, and if 1-class patterns were used as well, the identification of 120 types. The number of sera



3-Dimensional Intersecting Scheme for 15 Pools.

Figure 1. Three dimensional "intersecting serum scheme", after Kenny et al. (33). There are 25 antisera in each pool which is represented by a slice of the cube at right angles to the axis.

in each pool would then be 15, i.e., one serum per pool for 1-class patterns, plus 14 sera per pool for the 7 x 15 of 2-class patterns available. The three dimensional scheme given as an example by Kenny et al. (33) is, in fact, a special case also of the LBM pools method when 3-class patterns are used in "three dimensions", but excluding the patterns that do not fit the cube, in the same way as the "intersecting scheme" of Schmidt et al. (19) excluded some 2-class patterns of the LBM method, as discussed earlier. If all 3-class patterns in two dimensions instead of three dimensions were used, fifteen pools should allow the identification of 455 types (!), but the number of sera in each pool would be very large, 91 to be precise (3 sera per pool for each of 30 3-class patterns in groups of 15, plus one serum per pool for a 3-class pattern in a group of 5; the break-down being obtained by dividing 455 by 15 which gives a quotient of 30 plus one remainder).

The present author has dealt in some detail with the report of Kenny et al. (33) because of the misunderstanding that seemed to have prevailed concerning the relationship between the "intersecting serum scheme" and the combination pools method. Investigators who pursue false trails may be so discouraged that they abandon the use of a method that should actually be of practical value. Fortunately, these authors eventually came to the same conclusion made subsequently by Schmidt et al. in 1971 (2), that the LBM pools method was more efficient. "Our present results suggest that rhinovirus identification by this scheme should be practical ... for example, if S (the number of antisera per pool) could be set at 30, then the 1, 2, 3 combinatorial scheme could be used and 92 viruses identified by eight pools of 29 antisera each" (Kenny et al. (33)).

Kenny et al. mentioned as a pre-condition the availability of very high titred antisera that had minimum cross-reactivity and little or no anticellular antibody, a condition already prescribed for using the LBM pools. Technical problems such as this can be solved, as has been shown in the application of the LBM antiserum pools method for identification of enteroviruses, and the benefits to be gained in achieving similar economics in the identification of rhinoviruses would be greater. In regard to the suggestion of Kenny et al. (33) that 8 pools should be used for identifying 92 types, the present author would suggest that 12 pools be used. 1-class and 2-class patterns would enable the identification of 78 types (12 plus 66), with 12 antisera per pool. The extension of this scheme with 3-class patterns to cover 12 additional types, bringing the total to 90 would contribute 3 additional antisera to each pool (total, 15 antisera per

pool), and its extension to cover 102 types would increase the number of antisera per pool to 18. This number of antisera would seem to be more practical than the 25 required in the scheme suggested by Kenny et al. in view of present technical achievements in producing high potency rhinovirus antisera. If even more types have to be covered, then it is recommended that the scheme be extended by increasing the number of pools which can be done without altering the patterns already assigned to virus types in the existing scheme, as described above.

Although the advocacy of the "intersecting serum scheme" by Schmidt et al. (19, 24) did much to bring to the attention of other investigators the advantages of combination pools, by trying to "simplify" the LBM antiserum pools method, some confusion was caused. Happily, the principle of the LBM antiserum pools method appears to have been grasped now, and its scope may be appropriately described as a method for the identification of Picornaviruses.

A paper, entitled "The LBM antiserum pools for Rapid Identification of Picornaviruses", elaborating these points, has recently been submitted to the American Journal of Epidemiology in which the report of Kenny et al. (33) appeared.

It is gratifying that the LBM antiserum pools method can be applied successfully for the identification of both the enteroviruses and the rhinoviruses and the study of the epidemiology of the picornavirus group as a whole will be much facilitated by the readiness with which investigators may identify virus isolates. Kenny et al. (33), indeed, suggested that serum pool schemes might be designed to include antisera to more than one group of viruses for identification of viruses isolated in a tissue culture system that might yield viruses classified in different groups. Rhinoviruses have been isolated from materials from which enteroviruses, rather, were expected, and vice-versa, and much unnecessary labour was undertaken in establishing "echovirus 28" as a new enterovirus type when it had been previously identified as rhinovirus type 1. The error might have been avoided if the virus isolate had been tested against antiserum pools containing antisera to both enteroviruses and rhinoviruses. The idea of a comprehensive antiserum pool system embracing really large numbers of virus types must remain theoretical for the time being, but technical advances may show that "large" is only a relative term.

In other virus classification groups the application of the LBM antiserum pools method does not seem to be helpful at present. For example, while there are a large number of arbovirus types, their identification by the complement-fixation test is a much simpler matter than if neutralization tests had to be employed. Moreover, antisera

used for complement-fixation tests are usually of low potency and are unsuitable for use in pools especially as high serum concentrations give rise to anti-complementary results.

Polyvalent sera, i.e., antiserum pools, have been used for long in the identification of bacteria, for example, salmonella. Though the antigens of these bacteria occur in combinations that enable identification of groups and occasionally of species (types), antisera are not pooled in combinations that would permit identification of types. On the contrary, much labour is involved in absorbing non-specific antibody from antisera.

Oakley and Warrack in 1953 (34) reported a method of combining in vivo and in vitro tests for routine typing of Clostridium welchii. Toxins produced by an organism were identified by the neutralization pattern obtained in neutralization tests of the toxic filtrate with antitoxins prepared against different types of Clostridium welchii. This method was improved upon by investigators at the Wellcome Research Laboratories who tested trypsin-treated filtrates and untreated filtrates against 7 antitoxin pools containing different combinations of antitoxins (plus two controls without antitoxin). The mixtures were then tested for neutralization of toxin by the intracutaneous method in guinea-pigs, and any of five toxins (A to E) present would be identified by the pattern of results obtained. The attention of the present author was drawn to the method of the Wellcome Research Laboratories which bears a resemblance to the LBM antiserum pools method when he came across it recently in Cruickshank's "Medical Microbiology", 11th Edition, 1965, (35) but he has been unable to trace an account of it in published scientific literature. It is interesting that the problem of identification of even as few as five bacterial antigens should have been solved by a method analogous to the combination antiserum pools for viruses.

So far the combination pools method has been concerned with the identification of antigens. An interesting application of the method may be of practical value for the identification of antibodies in certain circumstances. In many studies only limited amounts of patients' sera are available and it would be of great advantage if combination pools of antigens could be prepared so that antibody for one of the antigens included in the pooling scheme could be detected in one experiment with a small number of aliquots of a patients' serum. For this approach to be successful, it is necessary that antibodies to the antigens concerned do not normally occur, otherwise the problem of "ambiguous" results due to "mixed antibodies" would be vexatious, and that the antigens may be pooled without causing heterotypic reactions. If such conditions are met, very much more information could be obtained from field serum samples,

especially if the method is combined with a microtechnique (32). If "multivalent antibodies" do occur to a limited extent they could be detected if the pooling system employs, exclusively, 2-class or 3-class patterns which would detect "antibody-mixtures" by the anomalous results obtained. The application of the combination pools method for the identification of antibodies is theoretically possible and would appear to be a good technique looking for a problem to be solved. Although it has been discussed entirely in the context of the neutralization test, its adaptation to the precipitation test and other antigen-antibody reaction should be considered.

ACKNOWLEDGEMENTS

The author is deeply grateful for help and encouragement given by Dr. Joseph Melnick in whose department the theme of this thesis was engendered. As Director of the WHO International Centre for Enteroviruses, Dr. Melnick perceived how LBM pools could facilitate the identification of enteroviruses and advocated the adoption of the method when to some it seemed unnecessarily complicated.

In the experimental work performed in the Department of Virology and Epidemiology, Baylor University College of Medicine (now Baylor Medical College), Houston, Texas, the author leaned heavily upon Dr. Matilda Benyesh-Melnick for he was quite innocent of knowledge of enteroviruses when he arrived in Houston and had to learn the techniques he used from her hands. Much of the materials used had been personally collected or prepared by Dr. Benyesh-Melnick, and many of the tests the author performed were repeated by her. Without her generous collaboration and patient guidance, the key publication of Lim and Benyesh-Melnick would never have seen the light of day.

The author's visit to Houston was made possible by a travel grant from the Rockefeller Foundation. Thanks are also due to the WHO for grants in support of the WHO Regional Centre for Enteroviruses in Singapore, located in the author's department, the Department of Bacteriology, University of Singapore.

The author has pleasure in thanking Miss Kok Him Ho for so carefully typing the manuscript and tables and the printer, Eurasia Press of Singapore, for the final publication and binding, so beautifully done.

Cup of kindness.

Once in a while, a man has the opportunity to achieve something he can be proud of. If he is wise, he will reflect that he was able to do so because he happened to be at the right place, at the right time, with the means to hand. All that went before and after belonged to others; his, was only a moment.

The author has pleasure in mentioning, with appreciation, others besides the above whose assistance some time, some place, delivered him one day at Fortune's door, Key in hand:–

The staff of the Department of Bacteriology, University of Singapore: Professor James H. Hale, formerly, Head of the Department, Dr. L. H. Lee, Dr. Y. C. Chan, Dr. Marguerite Yin-Murphy, Dr. P. H. Chee, now in private practice and Dr. W. O. Phoon, now Professor of Social Medicine and Public Health, University of Singapore, his

associates; Mr. R. Manikam, Mr. K. Panjanathan, Mr. K. Kanapathipillai, Mr. J. J. Pereira, Mr. K. S. Chew, Mr. Benny Soh and Mr. Leonard Chee (Senior Technicians and Technicians); Mrs. A. Samuel and Miss Kok Him Ho (Secretaries);

Professor J. C. Cruickshank and Professor E. T. C. Spooner, London School of Hygiene and Tropical Medicine; Professor Wilson Smith, formerly Professor of Bacteriology, University College Hospital Medical School and Dr. (Mrs.) Margaret Sabine (Edney), formerly Research Fellow at this School (1954); Professor Sir MacFarlane Burnet, formerly, Director of the Walter and Eliza Hall Institute of Medical Research, Melbourne and Dr. E. L. French, formerly, Virologist at the Institute, (1956); Dr. Carleton D. Gajdusek, of the NINDS, National Institutes of Health, Bethesda, Md., U.S.A.; Professor Lim Tay Boh, formerly Vice-Chancellor, University of Singapore (1966).

It is not out of place in this work to conclude with a tribute to the author's alma mater and the fair city of Edinburgh where 'twixt Jamieson and Davidson, he was wont to indulge in billiards as well as to explore the mysteries of predicting the outcome of football matches. There is a resemblance, it may be perceived, between the object of this exercise and the procedure adopted by followers of that popular British pastime of combining ("permuting" is the term they used, but incorrectly) their predictions to cover specified alternatives. That the author's youth was not entirely mis-spent may be attributed to kindness of Sir John Fraser under whose tolerant eye he spent many happy hours playing a small part in experiments on the administration of penicillin by the Eu-drip method (McAdam, I.W.J., Duguid, J.P., Challinor, S.W. Systemic administration of Penicillin. Lancet, 2:336-338, 1944). The taste for tinkering at the bench, then acquired, has led the author by devious paths to his present occupation.

REFERENCES.

1. Lim, K.A., and Benyesh-Melnick, M.

   Typing of Viruses by Combinations of Antiserum Pools. Application to Typing of Enteroviruses (Coxsackie and ECHO). J. Immunol., 84 : 309-317, 1960.

2. Schmidt, N.J., Melnick, J.L., Wenner, H.A., Ho, H.L., and Burkhardt, M.A.

   Evaluation of Enterovirus Immune Horse Serum Pools for Identification of Virus Field Strains. WHO Bull. 45 : 317-330, 1971.

3. Committee on the Enteroviruses.

   The Enteroviruses. Am. J. Pub. Health, 47: 1556-1566, 1957.

4. International Enterovirus Study Group.

   Picornavirus Group. Virology, 19: 114-116, 1963.

5. Horstmann, D.M.

   The Picornavirus Group. Chapter 17 in "Viral and Rickettsial Infections of Man", 4th Ed. Editors, Horsfall, F.L., and Tamm, I. J.B. Lippincott, Co. Philadelphia, 1965.

6. Bodian, D., and Horstmann, D.M.

   Polioviruses. Chapter 18 in "Viral and Rickettsial Infections of Man", (see Ref. 5).

7. Dalldorf, G., and Melnick, J.L.

   Coxsackie Viruses. Chapter 19 in "Viral and Rickettsial Infections of Man", (see Ref. 5).

8. Melnick, J.L.

   Echoviruses. Chapter 20 in "Viral and Rickettsial Infections of Man", (see Ref. 5).

9. Melnick, J.L.

   Enteroviruses : A Review of their History and Present Status. Israel J. Med. Sci., 1 : 272-283, 1965.

10. Melnick, J.L., and Wenner, H.A.

    Enteroviruses. Chapter 17 in "Diagnostic Procedures for Viral and Rickettsial Infections" 4th Ed. Editors, Lennette, E.H., and Schmidt, N.J. Am. Pub. Health Association, Inc. 1969.

11. Melnick, J.L.

    Classification and Nomenclature of Animal Viruses, 1971. Progr. Med. Virol.,

13 : 462-484, 1971.

12. American Public Health Association.
Diagnostic Procedures for Virus and Rickettsial Diseases. 2nd Ed. Editor, Francis, T. Am. Pub. Health Association, New York, 1956, pp. 94-95.

13. Melnick, J.L.
Problems Associated with Viral Identification and Classification in 1956. Ann. N.Y. Acad. Sci., 67 : 363-382, 1957.

14. Swain, R.H.A., and Dodds, T.C.
"Clinical Virology". E. & S. Livingstone, Ltd., Edinburgh and London, 1967, p. 174.

15. Hoskins, J.M.
"Virological Procedures". Butterworths, London, 1967, pp. 194-195.

16. Yow, M.D., Melnick, J.L., Phillips, C.A., Lee, L.H., South M.A., and Blattner, R.J.
An Etiologic Investigation of Infantile Diarrhea in Houston during 1962 and 1963. Am. J. Epidem. 83 : 255-261, 1966.

17. Nair, E., and Kalra, S.L.
Cytopathogenic Enteroviruses in Delhi Area. I. From Cases of Diarrhoea and Healthy Controls. Indian J. Med. Res. 57 : 141-148, 1969.

18. Feldman, R.A., Christopher, S., George, S., Mamath, K.R., and John, T.J.
Infection and Disease in a Group of South Indian Families. III. Virological Methods and a Report of the Frequency of Enteroviral Infection in Preschool Children. Am. J. Epidemiol. 92 : 357-366, 1970.

19. Schmidt, N.J., Guenther, R.W., and Lennette, E.H.
Typing of ECHO Virus Isolates by Immune Serum Pools. The "Intersecting Serum Scheme". J. Immunol. 87 : 623-626, 1961.

20. Desai, R.M., Jhala, H.I., Dave, K.H., and Gharpure, P.V.
Identification of Non-polio Human Enteroviruses Isolated from Healthy and Sick Children in Bombay. Indian J. Path. Bact. 8 : 297-303, 1965.

21. Yin-Murphy, M., and Lim, K.A.
Enteroviruses in Singapore. Singapore Med. J. 9 : 252-256, 1968.

22. Moritsugu, Y., Komatsu, T., Karasawa, T., Hara, M., Tsuchiya, K., Tsuchiya, Y., Nakao, C., Soda, K., and Tagaya, I., Miki, Y., Komiyama, S., Miztani, H., Yamazaki, Y., and Asada, S. A Longitudinal Study of Enterovirus Infections in Kawasaki, Japan. I. Virus Isolations from 1962 to 1966. Japan J. Med. Sci.

Biol. 23 : 147-160, 1970.

23. Melnick, J.L., Wenner, H.A., and Rosen, L.

The Enteroviruses. Chapter 5 in "Diagnostic procedures for Viral and Rickettsial Diseases". 3rd Edition. Editors, Lennette, E.H., and Schmidt, N.J. Am. Pub. Health Association, Inc. New York, 1964.

24. Schmidt, N.J.

Tissue Culture methods and Procedures for Diagnostic Virology. Chapter 3 in "Diagnostic Procedures for Viral and Rickettsial Diseases", 3rd Edition (see Ref. 23).

25. Wenner, H.A.

Outline of Laboratory Procedures for the Diagnosis of Enteroviruses Infections. Chapter 6 in "Diagnostic Procedures for Viral and Rickettsial Diseases", 3rd Edition (see Ref. 23).

26. Hampil, B., Melnick, J.L., Wallis, C., Brown, R.W., Braye, E.T., and Adams, R.R., Jr.

Preparation of Antisera to Enteroviruses in Large Animals. J. Immunol. 95 : 895-908, 1965.

27. Melnick, J.L., and Hampil, B.

WHO Collaborative Studies on Enterovirus Reference Antisera. WHO Bull. 33 : 761-772, 1965.

28. Hampil, B., and Melnick, J.L.

WHO Collaborative Studies on Enterovirus Reference Antisera. Second Report. WHO Bull. 38 : 577-593, 1968.

29. Melnick, J.L., and Hampil, B.

WHO Collaborative Studies on Enterovirus Reference Antisera. Third Report. WHO Bull. 42 : 847-863, 1970.

30. Hampil, B., and Melnick, J.L.

WHO Collaborative Studies on Enterovirus Reference Antisera. Fourth Report, 1972 (in preparation).

31. Hamparian, V.V., Leagus, M.B., and Hilleman, M.R.

Additional Rhinovirus Serotypes. Proc. Soc. Exp. Biol. Med. 116 : 976-984, 1964.

32. Gwaltney, J.M.

Micro-neutralization test for Identification of Rhinovirus Serotypes. Proc.

Soc. Exp. Biol. Med. 112 : 1137-1141, 1966.

33. Kenny, G.E., Cooney, M.K., and Thompson, D.J.

Analysis of Serum Pooling Schemes for Identification of Large Numbers of Viruses. Am. J. Epidemiol. 91 : 439-445, 1970.

34. Oakley, C.L., and Warrack, G.H.

Routine Typing of Clostridium welchii. J. Hyg. 51 : 102-107, 1953.

35. Cruickshank, R.

Medical Microbiology. 11th Edition. E. & S. Livingstone Ltd., Edinburgh and London, 1965, pp. 313-314.

The End.

TYPING OF VIRUSES BY COMBINATIONS OF ANTISERUM POOLS. APPLICATION TO TYPING OF ENTEROVIRUSES (COXSACKIE AND ECHO)

K. A. LIM AND MATILDA BENYESH-MELNICK

From the Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas

Reprinted from THE JOURNAL OF IMMUNOLOGY
Vol. 84, No. 3, March, 1960

TYPING OF VIRUSES BY COMBINATIONS OF ANTISERUM POOLS. APPLICATION TO TYPING OF ENTEROVIRUSES (COXSACKIE AND ECHO)[1]

K. A. LIM[2] AND MATILDA BENYESH-MELNICK

From the Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas

Received for publication July 14, 1959

In a classification scheme in which the prototype viruses do not cross-react with sera against heterologous types, identification of an unknown virus with a prototype is given by one positive result when the unknown is tested against immune sera to the prototypes. Where the number of established prototypes renders individual tests against all the prototype sera cumbersome, the conventional procedure is to screen the unknown virus first against pools of several sera. A positive result obtained against one of the pools leads to identification of the virus when it is tested against each member serum of that pool (1).

This procedure can be curtailed if the typing sera are distributed in serum pools according to a combination pattern so that each serum is included in different sets of one, two or more pools. A virus tested against the pools is identified in one operation by the pattern of positive results obtained.

The first part of this paper describes how such "combination pools" can be made. The second part presents results obtained when the method is applied to the typing of enteroviruses of the Coxsackie and ECHO groups by neutralization tests in tissue culture.

I. CONSTRUCTION OF COMBINATION POOLS

Figures 1–3 show distributional patterns for schemes employing four, five or seven pools.

In the first scheme (Fig. 1), 14 prototype sera are distributed in four pools so that the pattern of positive results obtainable against any type would be specific. The 14 patterns are derived by taking four items one at a time, then two at a time, then three at a time, giving four, six and four patterns respectively. The prototype serum 1 is distributed to pool A alone and the prototype serum 5 to pools A and B and so on. Should a virus when tested against the four pools give a positive result against pool A alone, it would be identified as type 1. Should it give positive results against pools A and B, it would be identified as type 5.

The diagnostic validity of a neutralizing pattern holds only in the absence of serologic cross-reactions and for a homogeneous test virus. Where an isolate contains more than one type of virus, the results may be ambiguous and lead to false interpretations. For example, in the four-pool scheme described, a mixture of type 1 and type 5 viruses would be neutralized by pool A and be indistinguishable from type 1 virus alone. A mixture of type 5 and type 8 viruses would also be neutralized by pool A, which contains antisera to both types, and the result falsely interpreted as due to type 1. These ambiguous possibilities arise only if the unknown virus specimen is not homogeneous and if in the scheme more than one class of distributional pattern is used. In a first class pattern each serum is distributed in one pool only, in a second class pattern, each serum is distributed in two pools, etc.

The 14 distributional patterns shown in Figure 1 exhaust the capacity of a four-pool scheme. The terminal patterns where a serum is distributed to none or all of the pools are omitted because it is desirable in practice that in a test the pools should give both positive and negative results. If more than 14 types are to be covered in a scheme the number of pools must be increased. The five-pool scheme shown in Figure 2 is analogous to the four-pool scheme. By increasing the number of pools to five the number of patterns available is increased to 30. However, the number of sera per pool is increased from 7 to 15. Since each serum in a pool is diluted by the other members, a pool of undiluted serum

[1] Aided by a grant from The National Foundation.

[2] Aided by a travel grant from the Rockefeller Foundation. Present Address: Department of Bacteriology, University of Malaya, Singapore.

Pattern	One-class				Two-class						Three-class			
Type serum	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Pools:														
A	×				×			×	×		×		×	×
B		×			×	×				×	×	×		×
C			×			×	×		×		×	×	×	
D				×			×	×		×		×	×	×
Result pattern	A	B	C	D	AB	BC	CD	AD	AC	BD	ABC	BCD	ACD	ABD

Figure 1. Distribution pattern for combination serum pools; (four-pool scheme for 14 types)

Pattern	One-class					Two-class									Three-class									Four-class				
Type serum	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Pools:																														
A	×					×				×	×			×		×			×	×	×		×		×				×	×
B		×				×	×					×			×	×	×			×	×	×		×		×				×	×
C			×				×	×			×		×		×	×	×	×					×		×				×		×
D				×				×	×			×		×			×	×	×		×			×	×				×	×	
E					×				×	×			×		×			×	×	×		×		×		×				×	×

Figure 2. Five-pool scheme for 30 types

Pattern.	One-class							Two-class																			Three-class					
Type serum.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Pools:																																			
A	×							×						×	×				×		×				×			×			×		×	
B		×						×	×							×		×		×						×			×		×		×	×
C			×						×	×					×		×					×		×			×		×		×		×	
D				×						×	×						×		×				×		×				×		×			×
E					×						×	×						×		×				×		×			×		×			
F						×						×	×					×		×				×		×			×		×			
G							×						×	×					×		×				×			×			×			×

Figure 3. Seven-pool scheme for 35 types

containing 15 members would find them mutually diluted 1:15. If, as is sometimes the case, high serum concentrations show nonspecific effects, and the technique demands that the serum concentration be not higher than 1:10, then the final dilutions of 15 members in a pool must give an average higher than 1:150.

If the potencies of the sera available do not permit such dilution, then the number of pools must be increased and the patterns used restricted to those of lower classes, for whereas five sera distributed by a one-class pattern contribute one serum to each pool, five sera distributed by a four-class pattern would contribute four sera to each pool.

The seven-pool scheme shown in Figure 3 uses only one-class, two-class and part of three-class patterns. There are 10 sera per pool and 35 types are covered. Part of this seven-pool scheme is used in the experiments described in part II of this paper.

In adopting any of the schemes described it would be desirable to leave unallocated patterns for the inclusion of additional types as yet not

established. If a three-class pattern were used the number of sera per pool would be increased by three for every seven additional types. Alternatively, the seven-pool scheme can be converted to an eight-pool scheme, for example, by distributing seven sera into a new pool H and one of these to each of the original seven pools. In this case, a two-class pattern would be used, and the number of sera in the original pools would be increased by one. These extensions do not alter the distribution of the original sera or significance of tests with the original types of viruses, a point which gives flexibility to the method.

II. TYPING OF ENTEROVIRUSES BY COMBINATION POOLS

Materials and Methods

Sera. The sera which appear in Tables I and II were, with the exceptions discussed in the following paragraph, reference monkey immune sera issued by the Committee on Enteroviruses, of the National Foundation (2).

Coxsackie A type 9 (A9) serum was rabbit immune serum prepared by Microbiological Associates, Bethesda. Coxsackie B types 1 through 5 (B1–5) were monkey immune sera prepared in our laboratory by immunization against mouse torso strains, and had been previously used in comparative tests with rabbit sera of Microbiological Associates. Their titers are available in the National Foundation's brochure (3). The sera appearing in Table IV were rabbit immune sera (3). Melnick's lact-albumin hydrolysate medium (M-H) was used as diluent for both sera and viruses.

Viruses. Prototype strains of the viruses used to test the serum pools are listed in the next to the last column of Table II. All had been passed in monkey kidney tissue culture. Fresh passages were used in these experiments.

Unknown viruses newly isolated from two sources were tested against the pools. Twenty-four were isolated from children in Mexico, who had been fed live poliovaccine, and from their families (4); and six were isolated in Houston from cases of aseptic meningitis or poliomyelitis and their contacts. These virus isolates were not neutralizable by polio sera types 1, 2 and 3 or a pool of all three in repeated experiments.

Neutralization tests. Neutralization tests were carried out by conventional methods in trypsinized primary monkey kidney tissue culture tubes (1, 2). Tubes were inoculated in triplicate. In all tests results were accepted if the dose of challenge virus used was in the range of 1.5–3.0 $\log_{10}$ TCD_{50}.

Combination serum pools. The antisera for 24 enterovirus types were distributed into seven pools (A to G), as shown in Tables I and II. The distribution pattern was derived from Figure 3. It was considered advantageous to omit one-class patterns from our scheme so as to reduce the number of ambiguous result patterns should more than one virus type be present in a test material. Consequently, any virus of the types included in the scheme should be neutralized by at least two pools and a neutralization obtained against one pool only would indicate heterogeneity of the virus tested. Polio types were excluded from the scheme because so many isolates in this laboratory prove to be polioviruses that it is more economical to test new strains first against polio sera rather than include them in a general diagnostic scheme. The final dilution of a serum in a pool was that recommended if the serum were to be used in a test

TABLE I

Composition of combination serum pool

Combination Pool	Antisera* Present in Pools								Number of Sera Per Pool
A	A9	E18	E2	E10	B1	E1	B3	E12	8
B	A9	E11	E5	E13	E19	B4	E3		7
C	E11	E14	E2	E6″	B2	B3	E8		7
D	E14	E15	E5	E7	B1	B5	E3	E12	8
E	E15	E16	E6″	E9	E19	E1	B3	E8	8
F	E16	E17	E7	E10	B4	E3	E12		7
G	E17	E18	E9	E13	B5	B2	E8		7

* A = Coxsackie A; B = Coxsackie B; and E = ECHO

TABLE II

Distribution of monkey typing sera in combination pools, and neutralization patterns obtained with prototype viruses

Type Serum*	Final Dilution of Serum	Serum Pools†							Prototype Virus Tested	Neutralization Pattern
		A	B	C	D	E	F	G		
A9	50	×	×						Grigg	AB
E11	50		×	×					Gregory	BC
E14	50			×	×				Tow	CD
E15	50				×	×			Charleston 96–51	DE
E16	50					×	×		Harrington	EF
E17	50						×	×	CHHE-29	FG
E18	50	×						×	Metcalf	AG
E2	100	×		×					Cornelis	AC
E5	100		×		×				Noyce	BD
E6″	32			×		×			D'Amori	CE
E7	100				×		×		Garnett	DF
E9	100					×		×	Bourne	EG
E10	100	×					×		Lang	AF
E13	100		×					×	Hamphill	BG
B1	32	×			×				Conn-5	AD
E19	50		×			×			Burke	BE
B5	200				×			×	Faulkner	DG
E1	200	×				×			Farouk	ABEG‡
B4	160		×				×		Texas-13	BF
B2	125			×				×	Ohio-1	CG
B3	320	×		×		×			Nancy	ACE
E3	500		×		×		×		Berardi	BDF
E8	500			×		×		×	Bryson	ACEG‡
E12	500	×			×		×		Travis	ADF
Number of sera in pool		8	7	7	8	8	7	7		
Serum concentration of pools		1:21	1:21	1:19	1:24	1:20	1:24	1:23		

* A = Coxsackie A; B = Coxsackie B; and E = ECHO.

† × indicates inclusion of serum in pool named at top of column.

‡ See text.

by itself. This was usually 20 units, a unit of serum being the dilution which would neutralize 100 TCD_{50} of virus (2). With sera of prototypes for which prime strains are known to exist, 100 serum units were used. This was done because prime strains require for their neutralization a higher concentration of the prototype serum (2).

In preparing the pools, a dilution of serum was first made which was 10 times stronger than the dilution recommended for typing. The appropriate "10 × concentrate" sera were pooled as indicated by Tables I and II, in 1 ml amounts, and each pool adjusted to a final volume of 10 ml with diluent. The resultant pools contained the sera in the required dilutions.

The order in which the sera appear in Table II originally corresponded with increasing titer of the sera as it was desired to distribute the high titered sera towards the end of the table evenly between the pools. However, the dilutions at which some sera had to be used were modified after preliminary experiments, and the dilutions

ECHO-2 and ECHO-10 viruses. The antisera to these four types were further distributed into four subpools; A3-1, A3-2, etc., as shown in Table III. The distribution pattern was again derived from taking the four sera two at a time, but the pairs, ECHO-2 and ECHO-10, and Coxsackie B1 and ECHO-1, were omitted as they had been tested for in the previous subpools. When M-2 was tested against the second subpools, it was neutralized by subpool A3-3, which contained the antisera to Coxsackie B1 and ECHO-2.

These experiments on artificial mixtures of viruses demonstrated an economical direct procedure for analyzing for pairs of viruses in a virus isolate.

The experiments described above were done with pools of monkey sera (except for the one Coxsackie A9 rabbit serum which generally had higher titers than the other rabbit sera available). An experiment was conducted, however, with the rabbit sera which had titers sufficiently high to be used in pools (3). Table IV shows the distribution of antisera to 14 types in pools according to the serum patterns of Figure 1 and to the prototype challenge viruses. In all cases, the neutralization patterns obtained were in accordance with the distribution of the homologous antisera. Experiments with rabbit sera were not pursued as the range of typing sera available was limited.

To test the application of the method, 24 viruses isolated in Mexico which could not be neutralized by polio sera were tested against the monkey serum pools A to G. Fourteen isolates were identified in a single test with the seven pools—eight as Coxsackie B5, three as ECHO-1 and one each as ECHO-14, ECHO-15 and ECHO-18. The identifications were subsequently verified by tests against the respective homologous serum alone. Representative results are shown in Table V.

Ten of the 24 isolates tested were not neutralized by any pool. For this there were four possible explanations: a) The virus preparation tested contained an excessive proportion of inactive virus. The latter could bind antibody and divert it from neutralization of the active virus upon whose titer the challenge dose was based. Such an experimental error might be corrected if a fresh passage of the virus were used. Interference with neutralization by inactive virus could be aggravated if the serum used had fallen in titer from that upon which the typing

TABLE IV

Distribution of rabbit sera in combination pools and neutralization obtained with homologous prototype viruses

Type Serum*	Final Serum Dilution	Pools				Neutralization Pattern vs Homologous Virus
		A	B	C	D	
A9	25	×				A
B5	25		×			B
E3	20			×		C
E14	20				×	D
E1	30	×	×			AB
E8	30		×	×		BC
E11	30			×	×	CD
E5	50	×			×	AD
E7	75	×		×		AC
E10	75		×		×	BD
E2	100	×	×	×		ABC
E6	100		×	×	×	BCD
E12	125	×		×	×	ACD
E9	150	×	×		×	ABD
Serum concentration of pools		1:11	1:10	1:10	1:11	

* A = Coxsackie A; B = Coxsackie B; and E = ECHO.

shown in Table II were those used in the experiments to be reported. The serum concentrations of the pools varied between 1:19 and 1:24, at which concentrations the sera did not cause apparent nonspecific effects. The reciprocal of the serum concentrations of a pool is equal to the sum of the reciprocals of the final dilutions of the members of the pool divided by the square of the number of members.

RESULTS

When the prototype viruses shown in Table II were tested against the pools, they were neutralized by the pools which contained the corresponding antisera. For example, Grigg virus (Coxsackie A9) had the neutralization pattern AB, *i.e.*, it was neutralized by pools A and B which were the only pools containing the homologous antiserum. Similarly Nancy virus (Coxsackie B3) had the neutralization pattern ACE. However, Farouk virus (ECHO-1) was neutralized by pools A and E, which contained the homologous serum, and by pools B and G. The latter results are attributable to the presence in pools B and G of ECHO-13 serum, the lot available having antibodies to ECHO-1 as well (2). Thus the neutralization pattern of ECHO-1 virus was ABEG. On the other hand, the ECHO-13 prototype virus tested was a new plaque-purified strain, recently obtained from Dr. Hammon's laboratory. Its neutralization pattern was BG.

Bryson virus (ECHO-8) was neutralized by pools C, E and G, which contained the homolgous serum, and by pool A. The latter result is attributable to the presence in pool A of ECHO-1 serum which is known to neutralize ECHO-8 virus (2). At the dilution used (1:500), ECHO-8 serum did not neutralize ECHO-1 virus, as shown by failure of pool C to neutralize Farouk virus. Thus the neutralization pattern for ECHO-8 was ACEG.

Tests against ECHO types 10, 17, 18 and 19 were incompletely carried out, but satisfactory results obtained with the other 20 prototypes were considered adequate evidence for the practical application of combination pools.

Because one-class patterns were not used in our scheme, neutralization of a virus preparation by one pool only would immediately indicate the presence of a mixture of viruses of the types in that pool. Trials were conducted with experimental mixtures of the prototype viruses to test the procedure for analyzing pairs of viruses that were neutralizable by one of the pools.

Equal parts of two viruses diluted to contain 100 TCD_{50} per 0.1 ml were mixed together. M-1 was a mixture of Coxsackie B1 and ECHO-1 viruses; M-2 was a mixture of Coxsackie B1 and ECHO-2 viruses. Both these mixtures were found to be neutralizable by pool A alone. Therefore, the eight sera in pool A were combined in 6 subpools, A1, A2, A3, etc., as shown in Table III. The distribution pattern was obtained by dividing the eight sera into four pairs arbitrarily, in the order in which they appear in Table I, and then combining the four pairs two at a time. Both M-1 and M-2 virus mixtures were now tested against pools A1, A2, etc., with results shown in Table III. M-1 was neutralized by subpools A3, A4 and A5, and since the antisera to Coxsackie B1 and ECHO-1 were the only ones distributed to all three pools, M-1 was demonstrably a mixture of these two viruses. M-2 was neutralized by subpool A3 only, and so could be a mixture of Coxsackie B1, and ECHO-1,

TABLE III

*Neutralization of virus mixtures by subcombination pools**

Virus Mixture	Pool A Sera in Subcombinations						Pool A3 Sera in Subcombinations			
	A1	A2	A3	A4	A5	A6	A3-1	A3-2	A3-3	A3-4
	A9, E18, B3, E12	A9, E18, E2, E10	E2, E10, B1, E1	B1, E1 B3, E12	A9, E18, B1, E1	E2, E10, B3, E12	E1, E10	E1, E2	E2, B1	E10, B1
Coxsackie B1, ECHO-1	−	−	+	+	+	−	ND	ND	ND	ND
Coxsackie B1, ECHO-2	−	−	+	−	−	−	−	−	+	−

* + Indicates neutralization of virus mixture, − indicates lack of neutralization and ND indicates test was not done.

TABLE V

Representative results of neutralization tests of Mexico and Houston isolates with combination pools

Source of Specimens	Virus Isolate No.	Neutralization Pattern	Identification
Mexico	1929	DG	Coxsackie B5
	2408	ABEG	ECHO-1
	1923	CD·	ECHO-14
	3177	DE	ECHO-15
	3295		Untypable
	3295*	ACE	Coxsackie B3
	2685	†	Untypable
	2685*	DG	Coxsackie B5
Houston	8-89	CG	Coxsackie B2
	8-139	BC	ECHO-11
	8-77	EF	ECHO-16

* Virus harvested from test against pool of polio 1, 2 and 3 sera.

† Poliovirus type 2 recovered from fluids in these tests.

dilution was calculated. b) The virus was of a type against which only low titer serum was available and thus was not suitable for inclusion in the pools (such as ECHO-4). c) The virus was of a type not yet established and, therefore, its serum was not included. Consideration should also be given to those viruses not in the enterovirus group but which may be isolated from the lower alimentary tract (adenoviruses). d) The virus preparation contained more than one virus type, the antisera to which were not together in any one pool.

From the nature of the experiment which provided the Mexican isolates, it was reasonable to expect a high frequency of isolates containing both a poliovirus which had been fed to the subjects (4) and some other virus normally circulating in the community. To meet this possibility, the isolates that were not typable on the first attempt were investigated further as follows. The culture fluids of virus growing in the presence of a pool of all three types of polio antisera were harvested, titrated and tested once more with pools A to G. This procedure served not only as a confirmatory test of the first attempt, but also as a test with freshly passed virus material. Representative results of the second attempt are also shown in Table V (strains 3295 and 2685). After passage through the polio sera, strain 2685 could be identified as Coxsackie B5, which apparently was a prevalent type among the Mexico isolates. However, the virus which grew in the presence of pools D and G (both of which contained Coxsackie B5 serum) was subsequently found to be polio type 2. It was evident that isolate no. 2685 was originally not typable because it contained both Coxsackie B5 and polio-2 viruses.

Isolate no. 3295, passed in the presence of polio antisera, was identified as Coxsackie B3. The virus, which in the first attempt grew in the presence of Coxsackie B3 antisera, *i.e.*, pools A, C and E, was re-examined and proved to be neutralized by Coxsackie B3 antiserum and not by polio sera. Isolate no. 3295 was, therefore, a "typable" virus not identified on the first attempt because of experimental error, or perhaps, because Coxsackie viruses like the ECHO viruses exist in nature as strains within a type but showing some degree of antigenic variation within the type. Strains of broader antigenicity than the prototype strain are more difficult to neutralize with prototype antisera and have been referred to as prime strains (2, 5, 6). In the same fashion two more isolates were typed further, one as Coxsackie B5 and the other as ECHO-18.

Six isolates (passed through polio sera) remain untypable by the pools, for the reasons already elaborated. These isolates were tested against the following sera individually: ECHO types 4, 20, 22, 23, 24 and 25; they were found not to be neutralizable by these sera. Opportunity was taken to test them at the same time against Hammon's high-titer serum prepared with a plaque purified strain of ECHO-13, also with negative results. These isolates are, therefore, not neutralizable by the sera available to us, or they contain unrecognized mixtures of virus.

Eight viruses isolated in Houston gave the following results: four were identified as Coxsackie B2, two as ECHO-11, and two as ECHO-16 (see Table V). All these identifications were verified by tests against homotypic antisera individually.

DISCUSSION

The method described, for using typing sera in combination pools, reduces the number of tests that have to be done to identify an unknown

virus. It can be applied successfully only if the antigens do not cross-react to any great extent with heterotypic antisera, and if the potency of the antisera permit their mutual dilution when mixed together. The higher the specificity and potency of the antisera and the greater the number of types to be considered, the more the economy that may be gained.

The reported experiments on the typing of Coxsackie and ECHO viruses demonstrate that it is possible to test a virus isolate against all established types using only seven pools of sera. The pools actually used covered 24 types that were available, but reference to Figure 3 will show that 35 types can be covered by seven pools of 10 sera each. If the titers of additional types were high enough to allow for 13 sera per pool, *i.e.*, using seven three-class patterns not shown, then the scheme could be extended to cover 42 types. Alternatively, the scheme can be converted to an eight-pool scheme as described earlier in this paper.

In the experiments reported, all antisera except one were monkey sera. A four-pool scheme covering 14 types and using rabbit sera which were generally of lower titers than monkey sera (3) was tested against some prototype viruses. The experiments with the rabbit sera indicate that, whereas results were consistent with the distribution of the sera, the high serum concentration (which was approximately 1:10) caused some nonspecific inhibition, in that all pools of sera delayed the growth of virus as compared to controls without serum.

The possibility of more than one virus being present in an isolate (7) was constantly kept in mind. Considering only the cases where two viruses are present in such proportions that 100 TCD_{50} of the isolate would contain demonstrable virus of both types, the 24 types covered in the scheme potentially could yield 276 pairs. Approximately half of these pairs would be neutralized by one or two pools in the seven-pool scheme used because their antisera happened to be together in those pools, but the rest would not be neutralizable. As the scheme does not use one-class patterns, neutralization by one pool only immediately indicates the presence of mixed viruses. Neutralization by two pools, however, may in some combinations give rise to false identifications. For example, a mixture of ECHO-3 and ECHO-12 viruses would be neutralized by pools D and F, the only pools to contain antisera to both types. Such a neutralization result would be interpretable as neutralization of ECHO-7 virus whose antiserum was distributed to pools D and F. In fact, any mixture of these three viruses would give the same neutralization result. So if any isolate were identified as ECHO-7, it would have to be verified by a test against homotypic serum alone, if one wished to rule out the possibility of a mixture giving false results.

These ambiguous possibilities arise only because both two-class and three-class patterns were used; as only a few of the latter were used, the number of ambiguous possibilities is small (readily discerned by inspection of Table II). Of the types identified only Coxsackie B2 had a neutralization pattern (CG) which theoretically could be simulated by a mixture of Coxsackie B2 virus and ECHO-8 virus (neutralization pattern: ACEG). All three identifications of Coxsackie B2 virus type were in fact verified by homotypic tests.

As the number of types in the enterovirus classification scheme increases, it becomes more and more impracticable for all laboratories to contemplate identification by conventional methods of the agents they isolate in tissue culture. There is a need, however, for the role which these viruses play in the etiology of disease to be studied as widely as possible. The gap between what is practicable and what is desired might be bridged by application of the method described. Provided that specific sera of adequate titers are available, it is possible for a central laboratory to supply other laboratories with pools of sera made and tested in bulk. Without the necessity of having to carry stocks of antisera to all types, any laboratory can type virus isolates by tests against seven pools of sera, interpreting their results by means of a diagnostic table. In view of the possibility of mixed virus specimens giving false identifications, a small number of monovalent sera may be stocked as a supplement to test certain identifications. Even these may be dispensed with in the study of specific outbreaks, for when a sufficient number of isolates are studied it should be apparent, from the types identified, which isolates might be mixtures of other types identified in the same study.

The experiments described and the discussion mainly concern the application of combination

pools by neutralization tests. Polyvalent serum pools have of course been used for screening purposes, *e.g.*, in Salmonella, but because of the cross reactions that exist, the application of a combination pool system would present difficulties even if absorbed sera are used. However, providing the conditions of specificity are observed, there seems no reason why the method may not be employed in other systems such as complement fixation or agglutination.

Halonen *et al.* (8) have recently described a method of typing ECHO viruses by complement-fixation tests in which virus isolates were tested against 14 typing sera. In all, the identification depended on one positive reaction observed. As described in the present paper, 14 types may be covered by a four-pool scheme; thus tests against four pools of sera can replace 14 separate tests. Since complement-fixation tests are relatively simple to perform, there may be no advantage for some in reducing the number of tests from 14 to 4, but if the number of types to be tested against were increased substantially (the ECHO types alone now number 28) and the number of isolates to be identified were many, there would appear to be an urgent need for finding a way to reduce the work involved.

SUMMARY

Individual tests of a virus antigen against an array of typing sera can be replaced by tests against a small number of pools of these sera. The serum pools yield combinations of results specific for each type according to the distribution of the sera in the pools. The procedure for constructing such "combination pools" is described.

Typing sera for 24 enteroviruses (18 ECHO and 6 Coxsackie) were distributed by this procedure into seven pools of sera. Prototype viruses tested by a single neutralization test against these seven pools gave results specific for each type. The results obtained by using these pools in typing new virus isolates are presented.

The method has been successfully applied to the typing of new virus isolates.

Acknowledgment. The authors are indebted to Dr. Joseph L. Melnick for making much of the experimental material available and for his discussions and encouragement during the course of this study.

REFERENCES

1. MELNICK, J. L., Ann. New York Acad. Sc., **67:** 363, 1957.
2. Committee on the Enteroviruses, Am. J. Pub. Health, **47:** 1556, 1957.
3. The National Foundation, Statement Concerning ECHO Virus Rabbit Antisera Prepared by Microbiological Associates, 1957. Brochure Available on Request.
4. BENYESH-MELNICK, M., MELNICK, J. L. AND RAMOS-ALVAREZ, M., *Live Poliovirus Vaccines*, Papers Presented and Discussions Held at the First International Conference on Live Poliovirus Vaccines. Pan American Sanitary Bureau, Washington, D. C., p. 272, 1959.
5. WIGAND, B. AND SABIN, A. B., Fed. Proc., **17:** 538, 1958.
6. MELNICK, J. L., Progress M. Virology, **1:** 59, 1958.
7. HSIUNG, G. D. AND MELNICK, J. L., Ann. New York Acad. Sc., **70:** 342, 1958.
8. HALONEN, P., ROSEN, L. AND HUEBNER, R. J., Proc. Soc. Exper. Biol. & Med., **98:** 105, 1958

ENTEROVIRUS IMMUNE HORSE SERUM POOLS

Table 1. Distribution of enterovirus immune sera in Lim Benyesh-Melnick pools and neutralization patterns for identification of viruses

Immune serum	Immune serum present in pools:								Neutralization pattern for identification of virus
	A	B	C	D	E	F	G	H	
echovirus 15	×								A
coxsackievirus A7	×	×							AB
coxsackievirus B1	×		×						AC
echovirus 33	×			×					AD
coxsackievirus B4	×				×				AE
echovirus 7	×					×			AF
echovirus 4	×						×		AG
echovirus 1	×							×	AH
echovirus 21		×							B
echovirus 2		×	×						BC
coxsackievirus B2		×		×					BD
poliovirus 2		×			×				BE
echovirus 19		×				×			BF
coxsackievirus A9		×					×		BG
echovirus 3		×						×	BH
echovirus 24			×						C
echovirus 6			×	×					CD
coxsackievirus B5			×		×				CE
poliovirus 1			×			×			CF
coxsackievirus B3			×				×		CG
echovirus 12			×					×	CH
echovirus 25				×					D
echovirus 13				×	×				DE
echovirus 14				×		×			DF
echovirus 16				×			×		DG
poliovirus 3				×				×	DH
echovirus 11					×				E
echovirus 18					×	×			EF
echovirus 17					×		×		EG
echovirus 22					×			×	EH
echovirus 27						×			F
echovirus 20						×	×		FG
coxsackievirus B6						×		×	FH
echovirus 31							×		G
echovirus 23							×	×	GH
coxsackievirus A16								×	H
echovirus 29	×		×			×			ACF
echovirus 5	×				×		×		AEG
echovirus 26		×		×		×			BDF
echovirus 9		×				×		×	BFH
echovirus 30			×		×		×		CEG
echovirus 32				×	×			×	DEH

Table 2. Distribution of enterovirus immune sera in intersecting serum pools and neutralization patterns for identification of viruses

Virus	Immune serum present in pools:													Neutralization pattern for identification of virus
	1	2	3	4	5	6	7	8	9	10	11	12	13	
poliovirus 1	×						×							1,7
poliovirus 2	×							×						1,8
poliovirus 3	×								×					1,9
coxsackievirus A7	×									×				1,10
cosackievirus A9	×										×			1,11
coxsackievirus A16	×											×		1,12
coxsackievirus B1	×												×	1,13
coxsackievirus B2		×					×							2,7
coxsackievirus B3		×						×						2,8
coxsackievirus B4		×							×					2,9
coxsackievirus B5		×								×				2,10
coxsackievirus B6		×									×			2,11
echovirus 1		×										×		2,12
echovirus 2		×											×	2,13
echovirus 3			×				×							3,7
echovirus 4			×					×						3,8
echovirus 5 [a]			×			×			×		×			3,9
echovirus 6			×							×				3,10
echovirus 7			×								×			3,11
echovirus 9			×									×		3,12
echovirus 11			×										×	3,13
echovirus 12				×			×							4,7
echovirus 13				×				×						4,8
echovirus 14				×					×					4,9
echovirus 15				×						×				4,10
echovirus 16				×							×			4,11
echovirus 17				×								×		4,12
echovirus 18				×									×	4,13
echovirus 19					×		×							5,7
echovirus 20					×			×						5,8
echovirus 21					×				×					5,9
echovirus 22					×					×				5,10
echovirus 23					×						×			5,11
echovirus 24					×							×		5,12
echovirus 25					×								×	5,13
echovirus 26						×	×							6,7
echovirus 27						×		×						6,8
echovirus 29						×			×					6,9
echovirus 30						×				×				6,10
echovirus 31						×					×			6,11
echovirus 32						×						×		6,12
echovirus 33						×							×	6,13

[a] Since E31 serum contained antibody of high titre against prototype E5 virus, E5 virus was also neutralized by pools 6 and 11.